# Factores implicados en la respuesta inmunitaria en el ciervo ibérico

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A mi abuelo

A mis dos chicos
Al más pequeño por el gran cambio que ha supuesto en mi vida y a mi alrededor
Al mayor, gracias por esa paciencia infinita, por esa ayuda, por estar siempre ahí

A mis padres, gracias por ese apoyo constante y por darme todo lo que soy
A mi pequeña ahijada, Amelia, a mis hermanos
y a toda mi familia, en especial a mi tía

# ÍNDICE

## Organización de la Tesis

El presente trabajo de tesis ha sido estructurado con un primer apartado de **introducción** seguido de la descripción de las **hipótesis y objetivos** que centran esta tesis. A continuación se exponen los cuatro capítulos que componen esta tesis y que responden a los correspondientes objetivos. Cada uno de estos capítulos consta de uno o varios trabajos, de los que se expone previamente un resumen en castellano.

El primero de los capítulos de la tesis, denominado "**Factores que intervienen en la respuesta dérmica a la inyección de antígenos**", se basa en tres artículos:

1. "Optimal dose and timing in phytohaemagglutinin skin-testing of deer"
Fernández-de-Mera IG, Höfle U, Vicente J, García A, Rodríguez O, Gortázar C
New Zealand Veterinary Journal 54(6): 357-359, 2006
2. "The effects of sex and age on phytohaemagglutinin skin-testing of deer"
Fernández-de-Mera IG, Vicente V, Höfle U, Rodríguez O, García A, Gortázar C
New Zealand Veteriary Journal. En prensa.
3. "Factors affecting red deer skin test responsiveness to bovine and avian tuberculin and to phytohaemagglutinin in Spain"
Fernández-de-Mera IG, Vicente J, Höfle U, Ruiz-Fons F, Gortázar G

El segundo capítulo, "**Relación entre condición nutricional y capacidad de respuesta antiparasitaria**", incluye el siguiente artículo:

4. "Faecal excretion of *Elaphostrongylus cervi* (Nematoda) in relation to experimental supplemental feeding in red deer (*Cervus elaphus*): Does nutritional condition improve anti parasite response?"
Fernández-De-Mera IG, Vicente J, Fierro Y, Gortázar C

El tercer capítulo, "**El factor genético: polimorfismo del MHC II en ciervo ibérico**", consta de dos trabajos:

5. "Major histocompatibility complex class II polymorphism in a hunter-managed isolated Iberian red deer (*Cervus elaphus hispanicus*) population"
Fernández-de-Mera IG, Vicente J, Pérez de la Lastra JM, Mangold AJ, Naranjo V, Fierro Y, de la Fuente J, Gortázar C

BMC Ecology. En evaluación.

6. "Major histocompatibility complex class II polymorphism in relation to infections and life history traits of management relevance in Iberian red deer"

Fernández-de-Mera IG, Vicente J, Mangold AJ, Naranjo V, Fierro Y, Kocan KM, de la Fuente J, Gortázar C

El último capítulo, "**Expresión diferencial de genes relacionados con respuesta inmune en ciervos infectados y no infectados, expuestos de forma natural a *Mycobacterium bovis***", incluye el siguiente artículo:

7. "Differential expression of inflammatory and immune response genes in mesenteric lymph nodes of Iberian red deer (*Cervus elaphus hispanicus*) naturally infected with *Mycobacterium bovis*"

Fernández-de-Mera IG, Pérez de la Lastra JM, Ayoubi P, Naranjo V, Kocan KM, Gortázar C, de la Fuente J

Developmental & Comparative Immunology. En prensa

Finalmente, un último apartado engloba la **síntesis** y las **conclusiones** suscitadas de los trabajos aquí presentados.

# Introducción

# Introducción

En esta tesis doctoral se aborda el estudio de algunos de los factores implicados en la respuesta inmunitaria en el ciervo (*Cervus elaphus* L., 1758). Se sabe que factores muy distintos afectan a la capacidad inmunitaria en mamíferos (Ekkel et al. 1995; Hanlon et al. 1994). La condición corporal está estrechamente relacionada con la respuesta inmune mediada por células T, lo que se ha medido en aves por medio de la intradermorreacción con fitohemaglutinina (Alonso-Álvarez y Tella 2001; Cheema et al. 2001). También la edad o el sexo del hospedador determinan una mayor o menor capacidad de respuesta a los patógenos, particularmente en especies poligínicas y muy dimórficas como el ciervo (Vicente et al. 2007a). Finalmente, el genotipo participa en la determinación de la mayor o menor capacidad de respuesta de un hospedador a los patógenos, bien sea por efecto de la variabilidad a nivel genómico o por el papel de genes concretos (Acevedo-Whitehouse et al. 2005).

Tal complejidad de factores resulta difícil de investigar, y requiere una perspectiva multidisciplinar. Por consiguiente, en esta tesis se aplican técnicas de campo y de laboratorio como la intradermorreacción, la parasitología, la genética poblacional y la genómica. Se utilizan como modelos micobacterias como la tuberculosis bovina (microparásito intracelular), y helmintos como el protoestrongílido *Elaphostrongylus cervi*, (macroparásito de ciclo indirecto), todo ello con el fin de aportar conocimiento científico a la interacción entre los citados patógenos y su hospedador el ciervo, mediada por la respuesta inmunitaria.

## 1. El ciervo ibérico (*Cervus elaphus hispanicus*)

### 1.1 Biología general

Clasificación taxonómica

Clase: Mamíferos

Orden: Artiodactyla

Suborden: Ruminantia

Familia: Cervidae

Especie: *Cervus elaphus*

Subespecie: *C. elaphus hispanicus*

Número de cromosomas: (2n)=68.

El ciervo, *C. elaphus*, es de origen euroasiático. Es una de las más de 40 especies de cérvidos distribuidas por todo el mundo. La subespecie que habita el suroeste de España es *C. elaphus hispanicus* (Erxleben 1777) y es una de las 13 subespecies repartidas por Europa, siendo ésta una de las de menor tamaño de Europa. Su tamaño actual podría ser el resultado de un proceso de reducción de tamaño corporal que ha tenido lugar a lo largo de miles de años, como adaptación al entorno mediterráneo, caracterizado por un largo período de estiaje (Soriguer et al. 1994).

Posee un pelaje con predominio del color castaño uniforme, con la zona ventral más clara y un escudo anal casi blanco. La cola es corta, de color oscuro. La fórmula dentaria del ciervo es: I0/3; C1/1; PM3/3; M3/3.

Al nacer, los cervatillos tienen un pelaje característico con fondo castaño y manchas blancas que permanecen durante los tres primeros meses de vida. El dimorfismo sexual es muy acentuado. El peso corporal medio es de 70 a 150 kg en machos adultos y de 50 a 100 kg en el caso de las hembras. En granjas y explotaciones cinegéticas con alimentación suplementaria es fácil superar estos límites.

A partir del primer año los machos desarrollan anualmente unas cuernas que utilizan en las luchas en el periodo de celo, y en cuyo desarrollo tendrán especial importancia aspectos genéticos y de alimentación. Las hembras carecen de cuernas y poseen dos pares de mamas en posición inguinal. El régimen alimentario de los ciervos es herbívoro. La alimentación incluye una amplia variedad de especies tanto herbáceas como arbustivas.

La estructura social es matriarcal, los machos y las hembras forman grupos separados, excepto en época de celo. Los ciervos son poligínicos. La madurez sexual en las hembras la pueden alcanzar en su segundo año de vida en función del peso alcanzado en la época de berrea. La madurez sexual en los machos se alcanza al año y medio de edad, aunque serán los machos más desarrollados los que primero cubran a las hembras.

El celo del ciervo o berrea dura unas tres o cuatro semanas, periodo que suele estar comprendido entre finales de agosto y principios de octubre, aunque el manejo humano, reintroducción, alimentación complementaria, caza, etc, puede alterar los ciclos naturales, siendo en este sentido cada vez más frecuente encontrar poblaciones con periodos de celo más largos de lo habitual (Soriguer 1994). La gestación dura entre doscientos treinta y doscientos cuarenta días, los partos se producen durante el mes de mayo y principio de

junio, coincidiendo con la época de mayor biomasa vegetal. La lactación de los gabatos dura hasta noviembre o diciembre (Carranza 2002).

## 1.2 Distribución

En la Península Ibérica existen restos fósiles del género Cervus con varios millones de años de antigüedad y *C. elaphus* está presente desde al menos el Pleistoceno superior (hace 120.000 años). De esta época también han sido encontrados restos de gamo (*Dama dama*) y corzo (*Capreolus capreolus*), lo que indica que la especie ya estaba consolidada (Carranza 2002; Cassinello en prensa).

La distribución original en la Península Ibérica pudo estar más localizada en la mitad suroccidental. La distribución actual es reflejo de la expansión natural de las últimas décadas y de las múltiples introducciones con fines cinegéticos, encontrándose hoy en día por casi todo el territorio peninsular, excepto la parte más occidental de Galicia y la costa levantina (Carranza 2002).

Actualmente, las mayores densidades de ciervos en España se dan en el centro y sur del país. En estas zonas los manejos son cada vez más similares a la ganadería extensiva, lo que podría afectar al estado sanitario de esta especie (Gortázar et al. 2006; Vicente et al. 2005b). Habría que destacar la abundancia de ciervo en zonas como la Cordillera Cantábrica, donde se realizaron repoblaciones entre 1952 y 1970, o también las reintroducciones en el Pirineo oscense y otras áreas montañosas de la mitad norte de la Península Ibérica. A partir de estas reintroducciones, el ciervo ha ido expandiéndose posteriormente de forma muy notable (Gortázar et al. 2000; Carranza 2002).

Hoy en día, el incremento de la demanda de caza se puede constatar con el aumento de las explotaciones industriales dedicadas a la producción intensiva de piezas de caza para su comercialización. Los traslados de ciervos están en auge, realizándose entre fincas, entre comunidades autónomas y también desde otros países europeos. Esto ha hecho que en España se hayan empezado a instalar desde 1980 un número considerable de granjas cinegéticas cuya producción esta orientada a la repoblación de cotos de caza mayor (Carranza 1999; Martínez 2000). Además de la existencia de estas granjas cinegéticas, muchos cotos capturan ciervos para venderlos a otras fincas. Una de las soluciones que proponen algunos gestores al problema que conllevan los cerramientos de las fincas es el sistema de cría mixto, es decir, producir animales de manera intensiva y bajo condiciones de manejo controlado, para que siendo individuos de calidad contrastada, sean trasladados y cazados posteriormente. Estos manejos, desafortunadamente, no siempre siguen criterios

sanitarios y de conservación suficientemente rigurosos. En particular, esto implica un importante movimiento de animales que no se someten a un control veterinario suficiente, ya que actualmente no existe una legislación que contemple de forma apropiada y completa estos aspectos que exija tomar medidas de control sanitario.

## 2. El sistema inmunitario

La eficacia de una respuesta inmune depende principalmente del lugar donde se haya producido la infección y del agente que la ha causado. Existen patógenos que invaden las células huésped y aquellos que no son capaces de hacerlo, por lo que el sistema inmune ha desarrollado una gran variedad de respuestas apropiadas para combatir cada tipo de patógeno, al mismo tiempo que mantiene la tolerancia a componentes propios del organismo.

### 2.1 Inmunidad innata y adquirida

Para combatir un patógeno lo primero que hace el sistema inmunitario es reconocerlo como tal. Para ello ha desarrollado dos tipos de mecanismos: innatos y adaptativos, cuya principal diferencia radica en las estructuras de reconocimiento del patógeno, ya que los mecanismos efectores de la destrucción son similares.

La inmunidad innata está basada en mecanismos inespecíficos de acción inmediata, carentes de memoria y encargados de combatir la infección en su inicio y durante las primeras fases con gran eficacia. Si este tipo de mecanismo no consigue eliminar el patógeno, al menos lo mantiene bajo control mientras se desarrollan los mecanismos adaptativos que requieren más tiempo. Las células encargadas de esta primera línea de defensa son los fagotitos (neutrófilos y monocitos) y macrófagos, que tienen receptores innatos para reconocer estructuras comunes a múltiples patógenos, llamadas PAMP ("*Pathogen Associated Molecular Pattern*"). Se incluye también en este grupo las llamadas células "*natural killer*" (NK), una población linfocitaria con actividad lítica pero que carece de receptor específico, y no necesita preactivación para lisar a la célula infectada.

La inmunidad adquirida está basada en mecanismos específicos. Estos mecanismos presentan memoria y los responsables son los linfocitos B y T. Mientras que los linfocitos B secretan anticuerpos que se unen específicamente al patógeno, los linfocitos T necesitan de un procesamiento previo del agente extraño, para posteriormente reconocerlo asociado a moléculas propias en otras células. Los linfocitos T tienen varias funciones: unos ayudan

a los linfocitos B en la producción de anticuerpos, otros ayudan a los macrófagos y otros destruyen directamente la célula infectada (Roit 1998).

## 2.2 Componentes del sistema inmunitario

El sistema inmunitario en vertebrados está formado por un conjunto de células encargadas de la defensa frente a agentes infecciosos externos, y otras patologías como los procesos neoplásicos. Estas células son los linfocitos, las células dendríticas del bazo, las células epiteliales de Langerhans y también células especializadas del timo, médula ósea y demás órganos linfoides. Las células se encuentran organizadas en tejidos concretos: bazo, nódulos linfáticos, placas de Peyer, amígdalas, timo y médula ósea. Una pequeña población de linfocitos y macrófagos puede localizarse en sangre y linfa circulante.

### 2.2.1 Sistema del complemento

El sistema del complemento está constituido por más de 30 proteínas que se encuentran en el plasma y en la superficie de muchas células. Parte de los factores del complemento potencian la inflamación y la fagocitosis y actúan produciendo la lisis de células y microorganismos. Existen tres vías para la activación de este sistema: la vía clásica, la vía alternativa y la vía de la lectina. En la activación del complemento se pone en marcha una serie de reacciones consecutivas en cascada, de tal forma que a partir de cada una de ellas se genera un producto activo que además de determinar que la reacción consecutiva prosiga, puede tener diferentes acciones biológicas importantes en la defensa del organismo. Las tres formas de activación confluyen en la ruptura enzimática de la molécula C3, que conduce a la formación del complejo de ataque a la membrana, un complejo lipofílico de proteínas plasmáticas que abre poros en la superficie celular y lleva a la lisis de las células.

La vía clásica del complemento se activa por la unión del complejo C1 a la región Fc de los anticuerpos que se han unido a antígenos pertenecientes a los microorganismos. Por su parte, la vía de la lectina se activa gracias a la unión del complejo conformado por la lectina (MBP) y las proteínas de serina asociadas a la MBP (MASP1 y MASP2) con los residuos de manosa presentes en la superficie de las células bacterianas. Finalmente, la vía alternativa se inicia por la unión covalente de una cantidad pequeña de C3b a los grupos hidroxilo de los carbohidratos y proteínas presentes en la superficie bacteriana; este C3b está disponible gracias a una ruptura continua del C3 en el plasma (Janeway et al. 2003).

### 2.2.2 Células presentadoras de antígeno

La célula presentadora de antígeno es la encargada de capturar los antígenos circulantes, internalizarlos y procesarlos de forma adecuada para que puedan ser reconocidos por los linfocitos T. La presentación del antígeno se hace a través de una molécula especial, a la cual se unen los fragmentos antigénicos procesados por la célula presentadora de antígeno. Esta molécula es el complejo mayor de histocompatibilidad (MHC) de clase II. Las células presentadoras de antígeno constituyen una población heterogénea y, según la forma de capturar el antígeno, se dividen en:

- Monocitos y macrófagos del torrente circulatorio
- Macrófagos de ganglios linfáticos y timo (Unanue 1984)
- Células de Langerhans (Stingl et al. 1978)
- Células de Kupfer (Rubinstein et al. 1987)
- Células dendríticas (Inaba et al. 1983)
- Células B

Las células dendríticas son de especial interés, ya que constituyen una de las más potentes poblaciones de células presentadoras de antígeno. Están involucradas en muchos procesos inmunitarios, como la activación de una respuesta T primaria, debido a la existencia en su membrana de altos niveles de moléculas coestimuladoras (Banchereau y Steinman 1998).

### 2.2.3 Linfocitos

Los linfocitos son células muy especializadas y responden a grupos concretos de antígenos. La capacidad de respuesta del linfocito existe de manera innata en la célula, incluso antes de darse el primer contacto con el antígeno, debido a la presencia en su membrana de receptores específicos para los determinantes antigénicos. Así, un linfocito individual posee una población de receptores idénticos, de modo que se diferencia de otros linfocitos, o clon de linfocitos, por la estructura de sus receptores y también por el rango de moléculas antigénicas que necesita como estímulo para iniciar una respuesta.

La capacidad de un organismo de responder a numerosos antígenos le viene dada por la existencia de un gran número de clones linfocitarios con distintos receptores para diferentes antígenos. Por tanto, la población linfocitaria está constituida por una colección muy heterogénea de células. Existen dos grandes poblaciones linfocitarias:

- Linfocitos B. Son los encargados de la respuesta inmune humoral. Se encuentran en la sangre y órganos linfoides. Se caracterizan por la presencia de inmunoglobulinas (Ig) en su superficie. Estas Ig son sintetizadas por la propia célula y se insertan en la membrana, donde actúan como receptores de antígeno. Esta población linfocitaria ejerce también como célula presentadora y la existencia de este tipo de receptor hace que su actuación sea mucho más específica que el resto de este tipo de células (Lanzavecchia 1990). Sin embargo, las células B también pueden actuar de manera inespecífica, presentando antígenos para los cuales no tienen Ig específica que los reconozca (Lanzavecchia et al. 1985). Además de su papel como célula presentadora de antígeno, los linfocitos B se definen clásicamente por ser los responsables de la producción de anticuerpos. Reconocen específicamente un determinado antígeno a través de sus inmunoglobulinas de membrana; una vez reconocido, se dividen y diferencian a células plasmáticas que segregan anticuerpos de la misma especificidad que la Ig de membrana. Los anticuerpos segregados se unen al antígeno, y a su vez a diversas células y proteínas del complemento que se encargan de neutralizarlo.
- Linfocitos T. Son los responsables de la respuesta inmune celular. Los patógenos solo son accesibles a los anticuerpos en la sangre y los espacios extracelulares, pero algunas bacterias y parásitos, y sobre todo los virus, se multiplican en el interior de las células, donde no son detectados por los anticuerpos. La destrucción de las células infectadas está mediada por los linfocitos T. Las células T son de origen hematopoyético y después de un proceso de maduración en el timo, migran a los órganos linfoides periféricos. A diferencia de los linfocitos B, las células T necesitan que el antígeno sufra una serie de modificaciones para poder reconocerlo, de modo que las proteínas antigénicas primero deben ser degradadas, y solo son reconocidos pequeños fragmentos peptídicos tras unirse a moléculas de MHC en la superficie de la célula presentadora. El receptor de membrana de las células T reconoce en las células presentadoras al complejo MHC/péptido y se une a él, tras lo cual se produce una señal de activación. Para efectuar sus actividades, ambos receptores requieren de la expresión de un complejo proteico llamado CD3, compuesto por 5 proteínas denominadas alfa, delta, épsilon, dseta y eta, que en presencia de interacción con el antígeno transduce señales a través de la membrana celular del linfocito. El receptor de la célula T reconoce al antígeno asociado a moléculas de histocompatibilidad clase I o II. Existen dos tipos fundamentales de linfocitos T,

caracterizados por la expresión, en general mutuamente excluyente, de moléculas CD4 y CD8 en la superficie celular. Así se distinguen linfocitos T cooperadores o helper (Th), que expresan la molécula CD4, y linfocitos T líticos o citotóxicos (Tc), portadores de la molécula CD8 en su membrana.

En rumiantes, particularmente en los animales jóvenes, existe una alta proporción de linfocitos T circulantes, aproximadamente un 50% (Hein et al. 1991; Wyckoff et al. 2002). A diferencia de lo que ocurre en otras especies como el hombre o el ratón, existe una población importante de linfocitos T gamma-delta. Aunque se desconoce el papel que desempeñan estas células en rumiantes, parece que una de las principales funciones de estos linfocitos podría ser el reconocimiento de células blanco alogénicas portadoras de moléculas de histocompatibilidad clase I, no clásicas y poco polimórficas. También se ha sugerido que estas células pueden reconocer moléculas de histocompatibilidad autólogas clase I presentes en las células blanco dañadas. Otras evidencias sugieren que las células gamma-delta tienen la capacidad de reconocer proteínas de stress micobacterianas en presencia de células presentadoras de antígeno autólogas, tales como los linfocitos B.

El papel principal de los linfocitos T CD4 es la secreción de citoquinas con funciones de ayuda diversa sobre otros tipos celulares. Estas citoquinas son indispensables para la multiplicación y diferenciación de linfocitos B y linfocitos citotóxicos. Según las citoquinas que se liberen, se han descrito dos fenotipos de linfocitos Th:

- Las células Th1, que producen preferentemente IL-2, IFN-γ y TNF-α, y están asociadas a fenómenos de respuestas inmunitarias mediadas por células.
- Las células Th2, que producen principalmente IL-4, IL-5, IL-10 e IL-13, destinada a ayudar a las células B en la producción de anticuerpos (Mosmann et al. 1986).
- Finalmente, se habla de un perfil Th0 cuando se producen los dos tipos de citoquinas citadas, aunque es discutido si realmente existe esta tercera población de Th o si es más bien una mezcla de linfocitos Th1 y Th2.

La función principal de los linfocitos T CD8 es interaccionar con la célula diana y destruirla. Esta interacción se da a través de su receptor que reconoce de forma específica antígenos asociados a moléculas de MHC clase I. Los linfocitos T citotóxicos son los encargados, mediante mecanismos de lisis específica, de proteger al organismo frente al ataque de virus y bacterias y frente a la aparición de células tumorales, frenando su

crecimiento. También son los principales causantes de rechazos de tejidos y órganos, además de estar implicados en fenómenos de autoinmunidad (Casares 2001).

## 2.3 Complejo mayor de histocompatibilidad

Una de las características más importantes de las células del sistema inmunitario es su capacidad para distinguir entre lo propio y lo extraño. En mamíferos este hecho se evidenció en experimentos de rechazo de tejidos entre cepas diferentes de ratones, fenómeno que se atribuyó a un grupo de genes denominado Complejo Mayor de Histocompatibilidad (MHC) (Yamazaki et al.1978).

Este grupo de genes se denomina H-2 en ratón y está situado en el cromosoma 17, en humanos se llama HLA y se localiza en el cromosoma 6, mientras que en el caso de los bovinos el MHC se conoce con el nombre de BoLA y está localizado en el cromosoma 23 (Tate 1997). Estos genes codifican tres clases de moléculas, clase I, II y III, aunque las funciones de histocompatibilidad y reconocimiento antigénico son llevadas a cabo por los genes de clase I y II, mientras que las moléculas de clase III son proteínas séricas que forman parte del sistema del complemento.

El MHC posee dos propiedades que hacen difícil que los agentes patógenos escapen a las respuestas inmunitarias. En primer lugar, el MHC es poligénico, es decir, existen diversos genes de MHC clase I y II que codifican proteínas con diferentes capacidades de unión. En segundo lugar, el MHC es altamente polimórfico, de tal forma que existen múltiples alelos para cada gen, de hecho son los genes más polimórficos que se conocen. (Yewdell y Bennink 1992)

### 2.3.1 Estructura

### MHC clase I

Las moléculas de MHC I se expresan en la superficie de todas las células excepto en neuronas. El MHC I está formado por una glicoproteína de membrana de 44 Kd que constituye la cadena pesada α, unida de forma no covalente a la cadena ligera β que tiene un peso de 12 Kd. La cadena α es altamente polimórfica, lo que hace que entre individuos de una misma especie existan diferencias en esta molécula. La cadena ligera es necesaria para la expresión en la superficie de las moléculas MHC I, así como para estabilizar su estructura. La síntesis de las moléculas MHC I se da en el retículo endoplasmático, donde se unen péptidos derivados del procesamiento de proteínas antigénicas. Las moléculas del MHC I presentan péptidos intracelulares citosólicos (virales). La expresión de moléculas de MHC I

está regulada por citoquinas, en particular por interferones derivados de las respuestas inmunitarias. Algunos virus tienen la capacidad de disminuir la expresión del MHC I, al igual que algunos tumores, lo que facilita su evasión al control del sistema inmunitario (Casares 2001).

### MHC clase II

El MHC II está compuesto por dos glicoproteínas transmembranales que forman la cadena ligera β y la cadena pesada α, que se mantienen unidas de forma no covalente. Las dos están codificadas por diferentes genes y son polimórficas. Durante su síntesis en el retículo endoplasmático están asociadas a una tercera cadena, la cadena invariante. Las moléculas del MHC II presentan péptidos extracelulares que han sufrido endocitosis. Al igual que en las moléculas de clase I, su expresión también está regulada por citoquinas, principalmente por los interferones (Swier et al. 1998).

## 2.3.2 Mecanismo de procesamiento y presentación al MHC

Las moléculas del MHC I y MHC II presentan antígenos en los macrófagos a las células T, y como cada molécula une un espectro diferente de péptidos, la presencia de diferentes loci hace posible que cada individuo esté equipado para presentar un amplio abanico de péptidos diferentes y por tanto responder a diferentes antígenos. Este polimorfismo es de crucial importancia en el reconocimiento de antígenos por células T, ya que estas reconocen el péptido unido a una determinada variante alélica de la molécula de MHC, lo que se denomina restricción por MHC (Zinkernagel y Doherty 1974).

Los agentes infecciosos se replican en dos compartimentos celulares: los virus se replican en el citosol, mientras que la mayoría de las bacterias patógenas y algunos parásitos se replican en el compartimento vesicular, es decir, en los fagosomas y endosomas de la célula. El sistema inmune utiliza diferentes estrategias para deshacerse de ellos. Los agentes infecciosos pueden llegar al compartimento vesicular de la célula de dos maneras. Algunas bacterias, como las mycobacterias, tienen la capacidad de invadir macrófagos y proliferar en las vesículas. Otras bacterias, de desarrollo extracelular, pueden producir toxinas y productos que son internalizados por endocitosis o fagocitosis por los macrófagos y otras células; o como en el caso del linfocito B pueden captarlos a través de sus inmunoglobulinas de superficie. Los linfocitos T son capaces de reconocer de qué compartimento celular proceden los agentes infecciosos mediante la presentación de los péptidos extraños sobre la superficie celular en moléculas diferentes. Los procedentes del

citosol se presentan sobre moléculas MHC de clase I y los provenientes de los endosomas sobre las de Clase II.

El MHC I generalmente se presentan proteínas del citosol, que pueden ser productos normales de la propia célula, de un virus ubicado en el interior celular o producto de un agente infeccioso bacteriano que ha infectado la célula.

El sistema MHC II suele presentar proteínas extracelulares que entran por fagocitosis, son transportadas en vesículas y degradadas en los lisosomas por enzimas proteolíticos. Los gérmenes de desarrollo extracelular que son ingeridos por los macrófagos, y aquellos que se multiplican en el interior de las vesículas de éstas células (como las micobacterias) son degradados por las proteasas presentes en las vesículas en peptidos que se unen a los MHC clase II, y posteriormente son presentados a los LT CD 4. Lo mismo ocurre con cualquier proteína extracelular que sea internalizada por las células. El descenso del pH en este compartimento, facilita la degradación de la proteína captada en fragmentos peptídicos. El lisosoma podrá entonces fusionarse con vesículas procedentes del retículo endoplasmático, donde se ha dado la síntesis del MHC II. La unión entre la vesícula y el lisosoma provoca la degradación de la cadena invariante del MHC II, quedando sus dos cadenas α y β con un surco accesible para la unión de los fragmentos peptídicos del procesamiento del antígeno capturado. El complejo MHC/péptido es transportado a la superficie celular, donde puede permanecer durante días, debido a la estabilidad de su unión, antes de ser reconocido por las células Th (Cresswell 1994).

### 2.3.3 MHC II en ciervo

En 1999, Lewin realizó una revisión completa sobre el MHC de los bóvidos (BoLA), con referencia específica a la organización genética, al polimorfismo y a la función de los genes de la clase II. La región BoLA es diferente al MHC de seres humanos y de ratón, debido a que, por medio de una gran inversión, se han movido varios genes de la clase II. Por lo tanto, el acoplamiento cercano de los genes de MHC y otros genes asociados al MHC en seres humanos y ratones no parece ser necesario para la función inmunológica normal. En ciervo ibérico se sabe que el MHC tiene dos sitios de expresión, y no uno como en muchos otros organismos.

#### Expresión de los genes del BoLA clase II y polimorfismo

Los bovinos expresan una pareja de haplotipos del gen DR (DRA y DRB3) y uno o dos pares del gen DQ. La secuencia de codificación del DRA es monomórfica, mientras que el

DRB3 tiene más de 70 alelos conocidos hasta ahora. En contraste, DQA y DQB son polimórficos, con aproximadamente 40 alelos caracterizados (Nomenclatura del BoLA: http://www2.ri.bbsrcac.nk/bola/). Alrededor de la mitad de los haplotipos comunes de la clase II parece tener genes DQ duplicados, donde ambos juegos de genes DQ se expresan. Esta duplicación, combinada con el polimorfismo de los genes DQA y DQB, tiene el potencial de aumentar la variación en la superficie de la célula debido al inter- e intra-acoplamiento de las cadenas α y β. Por lo tanto, es probable que todos los bovinos expresen diferentes genes de la clase II, lo que puede contribuir positiva o negativamente en la respuesta inmune a un antígeno en particular.

Existen muchas diferencias dentro del MHC de las diferentes especies. Así por ejemplo, el gen DYA del BoLA no tiene ningún equivalente (ortólogo) humano (Andersson et al. 1988). Se ha identificado este gen DYA en bovino, ovino y caprino, pero no en cerdos, primates o roedores, lo que sugiere que el DYA se originó por la duplicación del gen después de la divergencia de suidos y de bóvidos. La región que contiene el DYA se denominó "clase IIb". Estos genes residen próximos al centrómero. La distancia física entre el DYA y los genes de la clase I es muy grande, situándose el DYA cerca del centrómero, y el resto del MHC cerca del centro del cromosoma. Por lo tanto, se deduce que hubo un cambio importante hasta llegar a la actual organización del BoLA.

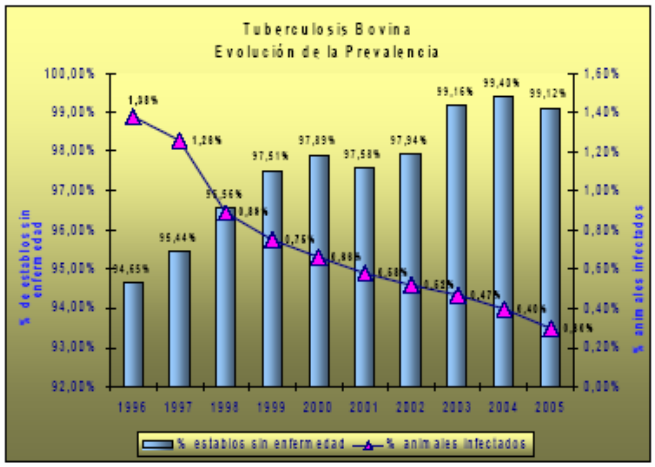
La pregunta que se surge es si esta organización tan radicalmente diferente del MHC bovino tiene un significado funcional. Se ha postulado que determinadas combinaciones de alelos que son importantes funcionalmente se han ido manteniendo por medio de la selección, incluyendo los genes implicados en el procesamiento antigénico. En este sentido, Casati et al. (1995) demostraron que la diferente respuesta inmune frente a *Mycobacterium bovis* se asociaba con determinados haplotipos del BoLA clase II.

Un logro importante para la investigación es entender cómo el polimorfismo del MHC contribuye a la resistencia y a la susceptibilidad a determinadas enfermedades, y al desarrollo de respuestas inmunitarias eficaces, lo que requiere un trabajo interdisciplinario, con estudios genómicos, moleculares, bioquímicos e inmunológicos. El aumento de la disponibilidad de herramientas para el mapeado del genoma bovino, proporciona una gama de oportunidades para mejorar nuestra comprensión en cuanto a las relaciones entre la estructura y la función de los genes del BoLA. Así, el análisis de la organización, de la estructura y de la secuencia génica dentro de diversos haplotipos del BoLA permite una comprensión más detallada de la evolución y de la función del MHC (Lewin et al. 1999).

## 3. Micobacteriosis en el ciervo ibérico

### 3.1 Situación epidemiológica

La tuberculosis en España es un grave problema, habiendo sido hace algunos años uno de los países de mayor tasa de infección dentro de la Unión Europea (Caffrey 1994; Liébana et al. 1995). La falta de éxito en las fases finales de los programas de erradicación en animales de granja ha sido relacionada en ocasiones con el mantenimiento de reservorios de la enfermedad en animales salvajes (Delahay et al. 2002; Caley y Hone 2004). En España, la situación de la ganadería bovina en cuanto a tuberculosis ha mejorado sensiblemente en la última década, pero ha alcanzado una fase asintótica en la cual conviene vigilar particularmente las especies ganaderas no saneadas y los posibles reservorios silvestres (Naranjo et al. en prensa).




Evolución reciente de las prevalencias de tuberculosis en ganado bovino en España. Fuente: MAPA

Las micobacterias, y en especial *M. bovis*, tienen un gran número de hospedadores (O´Reilly et al. 1995; De Lisle et al. 2001). El ciervo en particular es sensible a las micobacteriosis, y tanto la tuberculosis como la paratuberculosis constituyen problemas a nivel mundial en la explotación comercial de este ungulado (Mackintosh et al. 2004). Entre las micobacterias que pueden afectar a los cérvidos en España destaca fundamentalmente el complejo *Mycobacterium tuberculosis*, incluyendo a los agentes causales de la tuberculosis bovina y caprina (Aranaz et al. 2004; Gortázar et al. 2005).

En España, las prevalencias más altas de ciervos con lesiones compatibles con tuberculosis se dan en el centro sur de la Península Ibérica, siendo la proporción media de ciervos con lesiones compatibles con tuberculosis del 10-15 % en esta región. En áreas situadas al norte de España, como Pirineos, Valle del Ebro o Sistema Cantábrico, no se han encontrado animales con lesiones compatibles con tuberculosis, siendo Ávila y Teruel las

provincias más norteñas donde se han identificado este tipo de lesiones con posterior confirmación por cultivo y PCR (Vicente et al. 2006a).

La zona centro sur de la península es la que presenta mayores densidades de ciervo y donde, desde las últimas décadas, se vienen incrementando los manejos, incluyendo el vallado de fincas, la suplementación alimentaria o los traslados de animales (Vicente et al. 2007a), lo que complica el estudio epidemiológico y probablemente contribuya a dificultar el éxito de los programas sanitarios de erradicación en ganado doméstico. La prevalencia de dichas lesiones está relacionada positivamente con la edad del animal, y también con la presencia de jabalí y con la agregación de éste en puntos determinados como comederos o charcas. Además, más de la mitad de los individuos que presentan lesiones compatibles con tuberculosis presentan un cuadro de lesiones generalizadas, hecho que aumenta igualmente con la edad pero es independiente del sexo (Vicente et al. 2006a). Se sabe que también las prácticas de manejo del monte pueden afectar a la agregación de hospedadores, y algunas características de los hábitat mediterráneos pueden incrementar la frecuencia y la probabilidad de la transmisión de la enfermedad (Vicente et al. 2007a).

Otras micobacterias, como *M. avium avium*, son diagnosticadas esporádicamente en cérvidos en España (con frecuencia en ciervos de granja importados de Escocia). La paratuberculosis, cuyo agente causal es *M. avium paratuberculosis*, ha sido citada en el ciervo en Austria (Deutz et al. 2005), Italia (Nebbia et al. 2000) y en la República Checa (Machackova et al. 2004), entre otros lugares de Europa. En Nueva Zelanda constituye un importante problema para la cría de ciervos en granja (Mackintosh et al. 2004). En España recientemente se han detectado casos clínicos en ciervos de granja, y los estudios serológicos sugieren una amplia distribución de esta micobacteriosis en rumiantes silvestres, que merece ser estudiada en mayor profundidad (Reyes et al. en evaluación).

## 3.2 Diagnóstico de las micobacteriosis

El diagnóstico de las micobacteriosis, de por sí complejo por las particularidades de estos patógenos, es aún más difícil en fauna silvestre por la relativa novedad de algunas técnicas en su aplicación a estas especies (ej. Griffin et al. 2004).

El diagnóstico rutinario en animales domésticos se lleva a cabo mediante la prueba de intradermorreacción o prueba de la tuberculina, consistente en la inyección subcutánea de un antígeno inactivado y posterior medición de la reacción defensiva en forma de inflamación. La realización de este tipo de pruebas en animales salvajes es difícil en condiciones naturales y su interpretación es comprometida en animales en semilibertad o

durante operaciones de captura y traslado. La prueba requiere mantener a los animales en cautividad hasta la realización de la lectura, que tiene lugar 72 horas después de la inyección del antígeno. Este tiempo de retención y los dos manejos con su correspondiente inmovilización física o química suponen una gran complejidad logística e implican riesgos para el operario y para el animal.

Las pruebas serológicas no están aún bien desarrolladas en el caso de la tuberculosis, ya que su sensibilidad es muy limitada. Por tanto, actualmente estas pruebas solamente sirven como complemento de otras técnicas. La situación es distinta en el caso de la paratuberculosis, ya que los test serológicos desarrollados permiten la detección de animales positivos con unos niveles de sensibilidad y especificidad aceptables. No obstante, apenas existen referencias sobre la aplicación de estos diagnósticos en especies silvestres, por lo que resulta complicado establecer los puntos de corte y realizar una correcta interpretación de los resultados (Reyes et al. en evaluación).

Lo más habitual es que la tuberculosis sea detectada durante la necropsia. Las lesiones compatibles con tuberculosis pueden teñirse con la técnica de Ziehl-Nielssen, que tiñe de color rosa las bacterias alcohol-ácido resistentes, pero el diagnóstico debe corroborarse mediante pruebas más específicas como aislamientos, técnicas inmunohistoquímicas o pruebas moleculares (PCR). En el caso de la paratuberculosis, la PCR puede realizarse también sobre heces.

### 3.3 Genómica funcional

La genómica funcional puede ser utilizada como una herramienta de estudio en las micobacteriosis. Tradicionalmente, ha asociado rasgos o características visibles (fenotipo) con el gen o los genes que los producían (genotipo). Cada célula del organismo tiene el mismo material genético durante toda su vida. Sin embargo, la expresión del gen (su actividad) varía de unas células a otras, de unos estadios de desarrollo a otros, en procesos normales o patológicos y en función de las condiciones ambientales. Sólo entendiendo los pormenores de la expresión génica entenderemos los procesos biológicos moleculares en los que intervienen los genes. Para ayudarnos con este propósito se aplica la técnica de los chips de DNA (Microarrays), que permite en un solo experimento el análisis de muchos genes, bajo diferentes condiciones experimentales. Además, un rasgo puede venir determinado por un gen o varios genes pueden determinar un solo rasgo. Si alteramos esos genes, se reflejará en la característica que expresan. Esta teoría ayuda a caracterizar la función de los genes.

### 3.3.1 Arrays

Los microarrays son una herramienta que está revolucionando el análisis genético desde finales de los años 80, al poder estudiar un número elevado de genes en un solo análisis. Los microarrays son una matriz bidimensional de material genético que permite la automatización simultánea de miles de ensayos encaminados a conocer en profundidad la estructura y funcionamiento de la dotación genética de un individuo, tanto en diferentes estados de desarrollo como durante procesos patológicos que afecten al animal. El microarray es una colección de ADN, consiste en un gran número de puntos correspondientes a moléculas de ADN ordenados sobre un sustrato sólido, de manera que formen una matriz de secuencias en dos dimensiones. Estos fragmentos de material genético pueden ser un gen distinto, y el conjunto de ellos es un chip. A estos fragmentos de ADN de una sola hebra inmovilizados en el soporte se les llama "sondas". Los chips se analizan añadiendo una copia complementaria de la población total de ARNm convertida a ácido desoxirribonucleico (ADNc), permitiendo que se hibride con los ADN del chip. El ADNc está marcado con sustancias con propiedades fluorescentes que pueden ser visualizadas en un escáner o lector computarizado que mide la fluorescencia relativa de los puntos con material génico. Si un gen fluorece más o menos en el tejido problema que en el de referencia, significa que dicho gen estuvo más o menos activo y que probablemente es importante para el proceso que se esté estudiando. Normalmente, el resultado son imágenes con puntos rojos si hay hibridación, si el gen de la muestra problema se expresa (presenta actividad), o verde si no la hay. Este método se aplica en el análisis de la expresión genética, detección de mutaciones y polimorfismos, secuenciación, seguimiento de terapia, toxicología de fármacos, diagnóstico molecular, diseño de fármacos, etc.

En especies salvajes hay muy poca información sobre la expresión diferencial de genes en infecciones micobacterianas naturales. El estudio y la identificación de genes diferentemente expresados en animales salvajes resistentes o susceptibles a la tuberculosis contribuirían al entendimiento de la resistencia e inmunidad protectora frente a las micobacteriosis que algunos individuos poseen. Una herramienta para ello sería caracterizar la expresión de genes de la respuesta inmune e inflamatoria utilizando la hibridación con microarrays en biopsias de ciervos naturalmente infectados con *M. bovis* y ciervos resistentes a la enfermedad y analizar así los mecanismos de patogénesis y de inmunidad frente a infecciones micobacterianas, lo que tendría una gran implicación en el control de la tuberculosis.

Hasta ahora, en ciervo se desconoce esta información, pero sí existen estudios en nuestro país con otro ungulado silvestre como es el jabalí (Naranjo et al. 2006; 2007). En jabalíes resistentes a la tuberculosis existen dos genes sobreexpresados, el componente 3 del complemento (C3) y el metilmalonil-CoA mutasa (MUT), genes que recientemente han sido asociados con la resistencia a la tuberculosis en ratón (Keller et al. 2004). En estudios genéticos en jabalí, MUT también ha sido asociado con la resistencia a la tuberculosis (Acevedo-Whitehouse et al. 2005). Ambos genes podrían contribuir a la resistencia de los jabalíes a la tuberculosis al modificar la inmunidad innata, limitándose la capacidad de la micobacteria de infectar y persistir dentro de los macrófagos.

## 4. Parasitosis del ciervo ibérico

Los procesos parasitarios son una de las principales causas de enfermedad citadas en los cérvidos europeos, siendo las nematodosis pulmonares e intestinales y las distomatosis hepáticas las más importantes (Chroust 1989). Sin embargo no siempre queda claramente demostrada la etiología parasitaria de estas muertes, y existen autores que sugieren que las infestaciones parasitarias elevadas son más bien una consecuencia de otros procesos o de deficiencias nutricionales (Sugar 1997, Knox et al. 2006).

Prácticamente todos los parásitos del ciervo presentan una ciclicidad en su prevalencia, intensidad de parasitación o tasa de excreción de formas reproductivas (huevos, larvas o quistes). En la mayoría de los casos las mayores tasas de infestación y/o excreción se dan en primavera. Las parasitaciones más altas suelen darse en ejemplares jóvenes o muy viejos, así como en condiciones de estrés por falta de recursos tróficos (Rossi et al. 1997).

La mayoría de las helmintiasis y todas las protozoosis del tubo digestivo son contraídas durante el consumo de vegetales herbáceos. Cuando los lugares de alimentación de un ungulado son compartidos por otras especies (silvestres o domésticas) existe normalmente un alto grado de solapamiento entre sus especies parásitas. En ocasiones se ha comprobado que la lucha contra las parasitosis del ganado doméstico ha dado resultados espectaculares en las prevalencias halladas en ungulados silvestres como el ciervo, así como en la mortalidad atribuida a parasitosis. Si las zonas de alimentación de los ungulados se hallan muy localizadas (comederos) o cuando las densidades poblacionales son elevadas, la probabilidad de infestaciones masivas aumenta (Chroust 1989).

Un animal deficientemente alimentado o estresado (celo, transporte, densidad excesiva, etc.) o con escasas defensas (jóvenes, viejos, final de la gestación, enfermos) será más propenso a infestaciones parasitarias graves. La calidad del medio, especialmente en lo que se refiere a la disponibilidad de recursos tróficos, es por tanto determinante en la gravedad de las enfermedades parasitarias del ciervo (Sugar 1997). Por este motivo los parásitos, particularmente los nematodos gastrointestinales, han sido utilizados como herramienta de monitorización sanitaria en poblaciones de ungulados silvestres (Eve y Kellogg 1977; Sugar 1997; Rossi et al. 1997).

Los parásitos del ciervo en Europa se pueden clasificar a primera vista de la siguiente forma (Boch y Schneidawind 1988):

**Protozoos**

Coccidios: Parásitos del intestino, del género *Eimeria*. También *Cryptosporidium*. Según especies y grado de parasitación pueden ser patógenos.

Género *Sarcocystis*: Forman quistes en la musculatura esquelética y cardíaca. Tienen muy escasa patogenicidad. Están presentes en la mayoría de los ciervos adultos.

*Toxoplasma gondii*: Parásito intracelular que aparece en el tejido nervioso, vísceras y músculo de muchas especies animales y tiene por hospedador definitivo al gato. Carece de importancia en el ciervo, pero es una zoonosis.

Hemoparásitos: *Babesia motasi, B. capreoli* y *Theileria ovis* entre otras, se han citado regularmente en ciervos centroeuropeos. Algunas especies, como *B. capreoli*, pueden excepcionalmente causar infecciones masivas con muerte y abortos. En el ciervo también se ha citado la parasitación por *Trypanosoma* sp. y por *Eperytrozoon ovis*.

**Helmintos (nematodos, cestodos y trematodos)**

Trematodos (duelas, principalmente parásitos del hígado)

• Fasciola hepática y género *Dicrocoelium* en hígado, así como *Paramphistomum cervi* en rumen.

Cestodos (helmintos segmentados)

• Fases larvarias: Cisticercos, cenuros, quistes hidatídicos.

• Adultos: Ténidos del género *Moniezia*.

Nematodos (vermes redondos)

• Nematodos pulmonares: Conjunto de especies que provocan bronconeumonías verminosas en rumiantes. El género más patógeno es *Dictyocaulus* (*D. viviparus, D. eckerti*).

• Nematodos meníngeos: *Elaphostrongylus cervi* es extremadamente común (Vicente y Gortázar 2001; Vicente et al. 2006b) y aparentemente poco patógeno en el ciervo ibérico.

• Nematodos gastrointestinales: Conjunto de especies que provocan las llamadas gastroenteritis parasitarias de los rumiantes. Su poder patógeno depende de la intensidad de parasitación y de los géneros implicados, siendo *Haemonchus* el más patógeno.

• Otros nematodos incluyen los filáridos parásitos del hígado (*Elaeophora elaphi*) y del tejido conjuntivo (*Onchocerca sp.*, *Setaria sp.*).

**Artrópodos**

Insectos:

• Hipobóscidos: Dípteros picadores provistos de alas por lo cual no permanecen unidos al cadaver.

• Oéstridos: Diferentes dípteros cuyas larvas se desarollan en las vías respiratorias altas (*Cephenemyia auribarbis, Pharyngomyia picta*) o tejido subcuáneo (*Hypoderma diana*).

• Pulgas y malófagos (*Cervicola meyeri*).

Ácaros:

• Sarna (*Sarcoptes scabiei*).

• *Neotrombicula autumnalis.*

• Garrapatas: *Hyalomma marginatum marginatum*, *Rhipicephalus (Boophilus) annulatus*, *R. Bursa*, *Dermacentor marginatum, Ixodes ricinus.*

## 4.1 Ectoparásitos (garrapatas)

Las enfermedades transmitidas por garrapatas son un buen modelo para el estudio de las interacciones entre animales domésticos y silvestres, ya que los ungulados domésticos y silvestres comparten las mismas especies de garrapatas y son susceptibles a la infección por numerosos patógenos de forma similar (Estrada-Peña et al. 2004; Ruiz-Fons et al. 2006). Los ungulados silvestres tienen un papel importante en el mantenimiento de las garrapatas, pudiendo ser un reservorio de patógenos.

Son numerosos los agentes patógenos transmitidos por garrapatas, así como las especies de garrapatas involucradas en su transmisión. En España existen, al menos, 10 especies diferentes de ixódidos que parasiten el ciervo ibérico, siendo las más frecuentes *Hyalomma marginatum marginatum, Ixodes ricinos, Rhipicephalus bursa* o *R. (Boophilus) annulatus*, esta última sólo ha sido identificada en la provincia de Cádiz (Ruiz Fons et al. 2006). Algunas de estas especies también parasitan al jabalí. Se sabe que *Hy. m. marginatum*

y *R. bursa* presentan un patrón inverso en cuanto a su frecuencia relativa mensual, coincidiendo las frecuencias relativas más altas de *Hy. m. marginatum* con las más bajas de *R. bursa* a lo largo del año, ambas especies se pueden encontrar durante todo el año en ciervo y en jabalí en el centro sur de la península, presentando más de un ciclo de vida por año. Todo esto habría que tenerlo presente para entender el papel de los ungulados silvestres en el mantenimiento de las infestaciones de garrapatas y a la hora de mejorar los programas de control sanitario en este campo (Ruiz-Fons et al. 2006).

### 4.2 Helmintos: *Elaphostrongylus cervi,* modelo de estudio

Este es un parásito altamente específico, coevolucionado y subletal. En el ciervo ibérico, la prevalencia y la abundancia de excreción de larvas del género *Elaphostrongylus* varían de forma inversamente proporcional a la condición física general (estado nutricional, capacidad inmune, etc) de los ejemplares. Por tanto, el estudio de estos parámetros puede tener utilidad para el seguimiento sanitario de las poblaciones naturales.

*E. cervi* pertenece a la familia Protostrongylidae (Nematoda: Metastrongyloidea), que incluye parásitos pulmonares cuyos adultos frecuentemente forman pequeños nódulos inflamatorios en la zona caudo-dorsal de los pulmones de rumiantes salvajes o domésticos (Anderson 2000). Esta familia también incluye otros parásitos meníngeos de los géneros *Elaphostrongylus* y *Parelaphastrongylus*, que se describen naturalmente en poblaciones autóctonas de cérvidos eurasiáticas y americanas respectivamente (Mason 1995). El genero *Elaphostrongylus* comprende tres especies: *E. rangiferi* Mitskevich 1958, en el reno (*Rangifer tarandus*); *E. alces* Steen, Chabaud y Rehbinder 1989, en el alce Europeo (*Alces alces*); y *E. cervi* Cameron 1931, en el ciervo rojo, el corzo (*Capreolus capreolus*) y el ciervo Sika (*Cervus nippon*) (Mason 1995).

*E. cervi* fue descrito por primera vez en España recientemente (Vicente y Gortázar 2001; Valcárcel y Romero 2002). También ha sido descrito recientemente su patrón estacional de excreción de larvas infectantes de primer estadío (Vicente et al. 2005a). Los parásitos meníngeos o extrapulmonares, como pertenecientes a la familia Protostrongylidae, necesitan un molusco terrestre como hospedador intermediario, así, en la Península Ibérica, las babosas podrían estar principalmente implicadas. El ciervo ingiere accidentalmente gasterópodos como hospedadores intermediarios que contienen estadios larvarios infestantes 3 (L3), capaces de llegar al torrente sanguíneo desde las paredes intestinales. Tras su paso y maduración en el espacio subaracnoideo (donde se pueden encontrar ejemplares subadultos), los ejemplares adultos de *E. cervi* se localizan

definitivamente en las fascias y tejidos conectivos vinculados a la musculatura esquelética, donde viven en grupos o parejas. Principalmente se encuentran en la musculatura del pecho, intercostal, axilas e ingle. En esta localización las hembras producen huevos que son liberados al torrente sanguíneo y alcanzan los espacios capilares de los pulmones donde el estadio larvario infestante 1 (L1) eclosiona, pasa al espacio alveolar, asciende por el árbol bronquial y es deglutido. Finalmente L1 se excreta a través de las heces del ciervo al medio ambiente, donde la larva es capaz de diseminarse activamente en medios húmedos (Boch y Schneidawind 1988; Handeland et al. 2000) y penetrar activamente en el hospedador intermediario (Rezac et al. 1994).

A pesar de su localización, estos parásitos son escasamente patógenos cuando la infestación ocurre sobre su especie hospedadora habitual, pasando habitualmente desapercibidos. Al igual que se da con otras especies parásitas, la patogenicidad de este parásito en especies hospedadoras no adecuadas ha sido causa de que exista gran interés en su diagnóstico, especialmente de cara a la exportación de ejemplares cervunos desde zonas endémicas a otras zonas libres de la parasitación y donde existen otras especies de ungulados autóctonos (Gajadhar et al. 1995).

La agregación de los animales, por ejemplo en torno a puntos de agua, está asociada positivamente con la prevalencia de ***E. cervi*** (Vicente et al. 2006b), lo que puede ser debido al aumento de la probabilidad de contacto con gasterópodos infectados. Dicha prevalencia está también asociada positivamente con la edad, y las intensidades tienden a ser mayores en machos que en hembras. La inmunidad adquirida podría tener aquí efecto, ya que en hembras se ha comprobado que existe un ligero descenso de la intensidad de parasitación con la edad (Vicente et al. 2006b).

En cuanto a los tratamientos antihelmínticos frente a este parásito, el uso de la ivermectina, derivado de la avermectina con un amplio espectro frente a muchos nematodos y artrópodos, está muy difundido entre algunos gestores de fincas. Sin embargo, un estudio sobre la eficacia del tratamiento con este compuesto frecuentemente utilizado demostró que, aunque es capaz de reducir el nivel de excreción, no conseguía la supresión total de excreción larvaria, por lo que los nematodos adultos seguían siendo viables y reproductivos (Rodríguez et al. 2006).

## 5. Antecedentes sobre respuesta inmunitaria, tuberculosis y parasitosis en el ciervo ibérico

### 5.1 Dicotomía en la asignación de recursos

Los recursos que se encuentran a disposición de los animales silvestres son limitados. Por ello, éstos han evolucionado bajo la presión que supone invertir los recursos de la forma más adecuada: ¿invertir en crecimiento y reproducción o invertir en capacidad inmune?

En el ciervo, y probablemente en otros mamíferos silvestres, el tamaño del bazo constituye un indicador de capacidad inmune (Corbin et al. 2007). El tamaño de esta víscera depende de la condición física de su portador, y puede verse afectado por la asignación diferencial de recursos a capacidad inmune por un lado, y a crecimiento y reproducción por otro (Vicente et al. 2007a).

En esta figura, tomada de Vicente et al. 2007a, se representa un diagrama con la conexión entre las variables estudiadas. Así, las flechas con línea continua representan los efectos positivos, mientras que las discontinuas corresponden a efectos negativos. U representa las variables latentes (varianza inexplicada de la variable a la que afecta la flecha). El diagrama muestra así la asignación diferencial de recursos a capacidad inmune por un lado y a crecimiento y reproducción por otro, lo cual presenta marcadas diferencias entre sexos y edades.

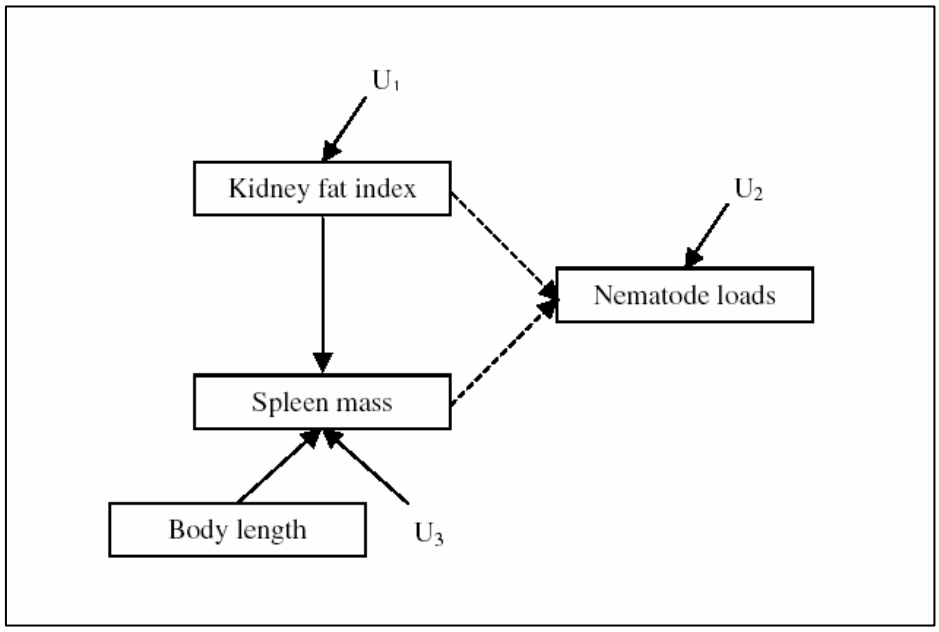



Figura tomada de Vicente et al. (2007a) en la que se representa la asignación diferencial de recursos

## 5.2 Dicotomía entre condición nutricional y riesgo de infección

Según lo explicado anteriormente, podríamos pensar que la mejora de la condición nutricional llevará a una menor carga parasitaria a través de una mejora en la capacidad de respuesta inmunitaria del hospedador. Sin embargo, cuando la citada mejora se procura a través de la alimentación suplementaria, el incremento de la agregación en torno a comederos (y por tanto de la probabilidad de la transmisión de patógenos) puede contrarrestar el beneficio esperado. Esto se ha observado en el caso de dos patógenos ampliamente distribuidos en ciervos del centro y sur de la península: *E. cervi* y el complejo *M. tuberculosis* (Vicente et al 2007b).

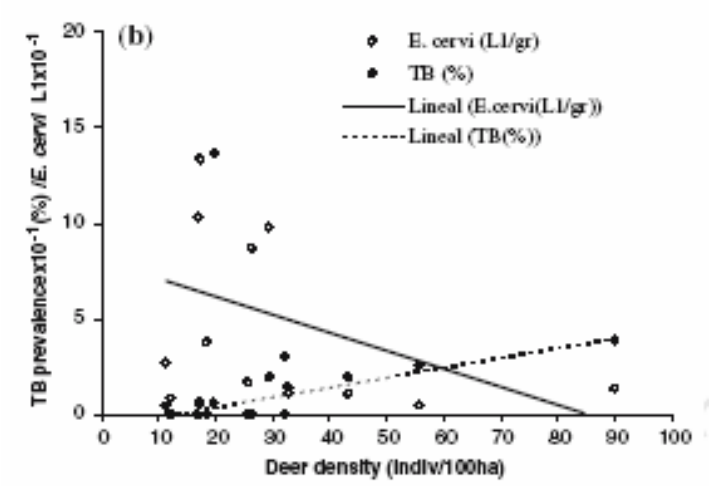



Figura tomada de Vicente et al. (2007b). A mayor densidad de ciervos, mayor prevalencia de lesiones compatibles con tuberculosis pero menor excreción de L1 de *Elaphostrongylus*. La contradicción se explica por la dicotomía entre condición nutricional, que mejora con la alimentación suplementaria en paralelo a la densidad, y riesgo de infección por TB, que aumenta con la agregación en torno a los comederos.

A efectos de gestión de las especies de fauna silvestre objeto de aprovechamiento cinegético, la recomendación que se deriva del estado actual de conocimientos es que debe procurarse mantener cargas ganaderas acordes con la capacidad de acogida del medio, y que los elementos que produzcan agregación espacial (comederos y puntos de agua) deben gestionarse con precaución. Desde el punto de vista científico, se requiere investigación aplicada para encontrar formas de aporte de agua y alimento compatibles con la sanidad animal, así como investigación básica para conocer mejor los complejos mecanismos mediante los cuales se produce la relación entre capacidad inmune, condición física y parasitismo.

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# Hipótesis y objetivos

# Hipótesis y objetivos de la tesis

La respuesta inmunitaria es la forma en que el cuerpo reconoce y se defiende a sí mismo contra las bacterias, virus y sustancias que parecen extrañas y dañinas para el organismo. Este término se utiliza para describir una serie de eventos dinámicos que ocurren in vivo, en el sistema inmune innato/constitutivo y adaptativo/inducible, originados por una alteración de la homeostasis de los tejidos.

El ciervo ibérico (*Cervus elaphus hispanicus*) constituye un buen modelo para estudiar las interrelaciones del triángulo recursos – hospedador – capacidad de respuesta. Utilizando como principales ejemplos la tuberculosis y el nematodo parásito *Elaphostrongylus cervi*, en esta tesis se plantea la hipótesis de que la capacidad de un individuo para controlar los citados patógenos está condicionada por factores fisiológicos como sexo, edad y condición física; y factores genéticos que modulan la resistencia o susceptibilidad a las infecciones.

En consecuencia, el objetivo general de esta tesis es aportar un mayor conocimiento sobre los factores fisiológicos y genéticos que modulan la respuesta inmunitaria celular y humoral en el ciervo ibérico, lo que se pretende mediante los siguientes objetivos parciales.

1º Evaluación de factores que intervienen en la respuesta dérmica a la inyección de antígenos, como los derivados purificados de proteinas micobacterianas (PPDs) o la fitohemaglutinina (PHA), un mitógeno derivado de *Phaseolus vulgaris*.

2º Experimentar con la relación entre condición física (estado nutricional) y capacidad del sistema inmunitario para controlar los parásitos, utilizando como modelo la excreción de parásitos protoestrongílidos.

3º Análisis del polimorfismo existente en el Complejo Mayor de Histocompatibilidad de clase II en una población de ciervo ibérico. Implicación en respuesta inmunitaria en cuanto a resistencia a determinados parásitos y enfermedades.

4º Estudio de la diferente expresión de genes relacionados con la respuesta inflamatoria e inmunitaria, analizando linfonodos mesentéricos de ciervos ibéricos infectados naturalmente con *Mycobacterium bovis*.

# Capítulo 1

## Factores que intervienen en la respuesta dérmica a la inyección de antígenos

**Determinación de la dosis óptima de PHA y tiempo hasta la lectura del incremento del pliegue cutáneo en la intradermorreacción en ciervo**
"Optimal dose and timing in phytohaemagglutinin skin-testing of deer"

**Efecto del sexo y la edad en el incremento de pliegue cutáneo en la intradermorreacción con PHA en el ciervo ibérico**
"The effects of sex and age on phytohaemagglutinin skin-testing of deer"

**Inyección intradérmica de PPDb, PPDa y PHA en el ciervo ibérico: Factores que afectan a la respuesta de la tuberculina**
"Factors affecting red deer skin test responsiveness to bovine and avian tuberculin and to phytohaemagglutinin in Spain"

## Determinación de la dosis óptima de PHA y tiempo hasta la lectura del incremento del pliegue cutáneo en la intradermorreacción en ciervo


### Resumen

En este trabajo se pretende establecer la dosis óptima de un mitógeno, la fitohemaglutinina (PHA), y el tiempo óptimo de lectura del incremento del pliegue cutáneo tras la intradermorreacción en el ciervo ibérico. Este mitógeno actuaría como indicador de la respuesta inmune mediada por células.

Para ello se utilzaron 20 hembras de ciervo ibérico a las que se inyectó intradérmicamente tres dosis de este mitógeno (10, 50 y 250 µg) en el lado derecho del cuello. En un cuarto punto también se inyectó como control un tampón salino fosfatado (PBS). El incremento de pliegue cutáneo se midió a las 0, 12, 24, 36, 48, 60, 72, 84 y 96 horas siguientes a la inyección.

La dosis más alta que se analizó (250 µg) fue la que originó una respuesta más clara y más duradera en el tiempo. En cuanto al tiempo de lectura, hubo muy poca variación en el incremento medido entre las 48 y 84 horas tras la inyección. Esta respuesta se correlacionó positivamente con el peso corporal del animal. La correlación del incremento del pliegue con el peso vivo no se apreció cuando se valoraron las dosis más bajas de PHA o el PBS.

Esta técnica podría ser fácil de aplicar en el campo de la investigación ecológica e inmunológica en ciervos, con un mínimo entrenamiento y sin requerir medios técnicos especializados en el ciervo.

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*Short Communication*

# Optimal dose and timing in phytohaemagglutinin skin-testing of deer

IG Fernández-de-Mera*, U Höfle*, J Vicente*†, A Garcia*, O Rodriguez* and C Gortázar*§


## Abstract

**AIM:** To establish the optimal dose of the mitogen phytohaemagglutinin (PHA) and the optimal time for measuring increased skin-fold thickness in red deer following intradermal injection, as an indicator of cell-mediated immune response.

**METHODS:** Three doses (10, 50 and 250 µg) of PHA were injected intradermally in the right side of the neck, and phosphate buffered saline (PBS) was injected at a fourth site as a control, in 20 captive Iberian red deer (*Cervus elaphus hispanicus*) hinds. Skin-fold thicknesses were measured at 0, 12, 24, 36, 48, 60, 72, 84 and 96 h following injection.

**RESULTS:** The highest dose of PHA tested (250 µg) resulted in a clear and long-lasting cellular response; increases in skin-fold thickness between 48 and 84 h post-injection varied minimally and response correlated positively with liveweight. No correlations with liveweight and no clear increases in skin-fold thickness occurred at the lower doses of PHA or the PBS.

**CONCLUSIONS AND CLINICAL RELEVANCE:** This technique could be applied with minimal training and without specialised equipment in deer, for immunological and ecological research.


**KEY WORDS:** *Cellular immunity, Cervus elaphus, mammalian immune response, wildlife bioindicators*

## Introduction

Measures of immune function have been recognised as valuable tools to study the behaviour, ecology and toxicology of wild species. While most of the techniques commonly used require specialised training or laboratory facilities, the phytohaemagglutinin (PHA) skin test is inexpensive and relatively easy to administer (Smits et al 1999).

PHA, a lectin from *Phaseolus vulgaris*, causes agglutination of erythrocytes, and growth, division, and non-specific activation of T-cells. It is routinely used *in vitro*. Lately, PHA has also been employed as a skin-test antigen, based especially upon its potential as a mitogen. The skin test comprises injecting PHA intradermally and measuring the proliferative response potential of circulating T-lymphocytes. The immune response is considered to be proportional to the difference in swelling between the site injected with PHA and a control site injected with PBS, or the increase in swelling before and after injection of PHA (Smits et al 1999).

In avian species, this technique has been routinely applied in poultry (Goto et al 1978), and nowadays is widely used as a measure of immune competence that has been shown to indicate individual fitness in ecology (Smits et al 1999). It could, therefore, be a relatively straightforward method to use *in vivo* to measure the immune capacity of mammals, but few studies using this technique in mammals have been reported. Apart from its use in laboratory animals, PHA has been used *in vivo* in domestic pigs, sheep and cattle (Kelley et al 1982; Ekkel et al 1995; Wambura et al 1998; de Groot et al 2001; Sevi et al 2001; Roberts et al 2002).

The PHA test has rarely been used in wild mammals. In wild populations of prairie voles (*Microtus ochrogaster*), an association between immune function and demography was shown (Sinclair and Lochmiller 2000). In wild boars, recent studies showed that the PHA skin reaction was positively correlated with the total number of adult parasites present (López-Olvera et al 2006). In deer, most literature deals either with *in vitro* tests (Griffin 1989) or different substances (Trindle et al 1979), especially mycobacterial antigens used for the diagnosis of tuberculosis and Johne's disease (Waters et al 2004). Sams et al (1996) examined the relationship between development, immunocompetence (PHA skin -testing), and tick burdens and neonatal mortality in an over-populated herd of white-tailed deer (*Odocoileus virginianus*). The risk of mortality up to 21 days of age was inversely related to delayed hypersensitivity to PHA.

Reliable methods of characterisation of the cellular immune reaction in mammals would improve our understanding of the involvement of many mechanisms of resistance against infections for both ecological and methodological research. Hence, we aimed to establish a protocol (dose and timing) for estimating the cell-mediated immune response in red deer to PHA *in vivo*.

## Materials and methods

### Study animals

An homogeneous group of 20 Iberian red deer hinds aged 3–5 years was kept on the experimental farm of the University of Castilla-La Mancha, Albacete, Spain. The animals were accustomed to handling and showed no outward signs of behavioural stress. They were individually identified with an ear tag and transponder. All hinds were kept together in an open-air enclosure during the study, which was conducted in January 2003. The deer were immobilised in an hydraulic crush for <5 min each, for injection and measuring skin thicknesses. Four areas of skin on the right side of the neck, each measuring 3 x 3 cm, were shaved prior to intradermal injection. On Day 1, the hinds were weighed to an accuracy of 100 g using a digital balance (Mettler-Toledo, Silla, Spain). All use of animals in this research was approved by the Research Ethics Committee of Castilla-La Mancha University, Albacete, Spain.


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| | |
|---|---|
| ANOVA | Analysis of variance |
| ICC | Intra-class correlation coefficients |
| PBS | Phosphate buffered saline |
| PHA | Phytohaemagglutinin |
| SD | Standard deviation |

### PHA skin test

The animals were injected intradermally at three separate locations with 10, 50 and 250 µg/ml PHA (Sigma-Aldrich, Missouri, USA), and one additional location with PBS, each separated by at least 4 cm; the volume injected was adjusted to 0.1 ml for all assays. One-ml syringes fitted with a 25-G ½-inch needle were used. Immediately prior to, and 12, 24, 36, 48, 60, 72, 84 and 96 h after, injection skin-fold thicknesses were measured twice, to the nearest 0.1 mm, using a digital calliper (Mitutoyo, Cardiff, UK), by the same person. In a separate study, a skin biopsy was taken from one hind 72 h after administration of 250 µg PHA. This tissue was fixed in buffered formalin, paraffin-embedded, routinely processed, and stained with haematoxylin and eosin, for histopathological examination.

### Statistical analysis

To test the effects of time and dose on increases in skin-fold thickness (as a response variable), a generalised linear mixed model (Glimmix procedure, SAS v9.1.3; SAS Institute Inc, Cary NC, USA) was used, with a factorial analysis of variance (ANOVA) structure which included time and dose of PHA as explanatory factors (and the two-way interaction), and animal as a random factor. A similar model was used for increases in skin-fold thickness at the site of injection of PBS, where the only explanatory factor was time. *Post-hoc* Tukey's tests were used to test differences between mean increases in skin-fold thickness at times 24, 36, 48, 60, 72, and 84 h post-injection. The reliability of double measures was assessed by calculating the intra-class correlation coefficients (ICC), whereby the closer the ICC is to one, the better the reliability. Spearman rank correlations were used to test for relationships between liveweight and increase in skin-fold thickness (at different doses, respectively; n=20) at all times post-injection. Statistical uncertainty was expressed through 95% confidence intervals of the mean.

## Results

Detectable increases in skin-fold thickness were evident in all deer injected with PHA and no aberrant reactions were seen. Change in skin-fold thickness over time for each dose of PHA used is shown in Figure 1. The effects of the dose ($p<0.001$) and time ($p<0.001$), as well as the interaction between these factors ($p<0.001$), were significant in the factorial ANOVA, which explained 62% of the total variability of the dependent variable, i.e. the change in skin-fold thickness over time varied between doses. Only the highest dose tested (250 µg) produced a clear increase in skin-fold thickness. *Post-hoc* comparison of means showed that, for this high dose, there were no significant differences between the mean increases in skin-fold thickness 24, 36, 48, 60, 72, and 84 h post-injection. The kinetics of response varied between animals; maximum responses were observed from 24 to 84 (mean 48) h post-injection (Figure 2). The greatest response, on average, was detected at 60 h, at which time the mean increase in skin-fold thickness was 6.14 (SD 1.92, range 3.35–9.25) mm. All responses measured between 48 and 84 h post-injection of the highest dose of PHA were greater than 2.0 (mean 5.75, SD 0.24, range 2.25–10.8) mm.

Increases in skin-fold thickness between 24 and 84 h post-injection of 250 µg PHA correlated with the liveweight of the hinds (Table 1). No correlation was found between increases in skin-fold thickness and liveweight at any of the lower doses of PHA, nor for the PBS control. No effect of time on the increase in skin-fold thickness was evident at the control site injected with PBS ($p>0.2$). At 72 h post-injection, the average change in skin-fold thickness at the site injected with PBS was 0.615 (SD 0.515, range –0.01 to 1.65) mm.

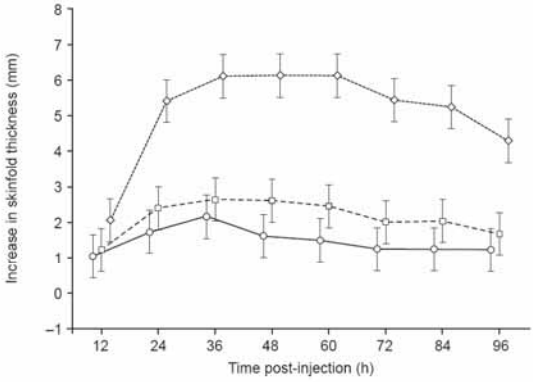


**Figure 1. Increase in cervical skin-fold thickness (± standard error) over time following intradermal injection with 10 µg (○), 50 µg (□), or 250 µg (◇) of phytohaemagglutinin in 20 Iberian red deer hinds.**

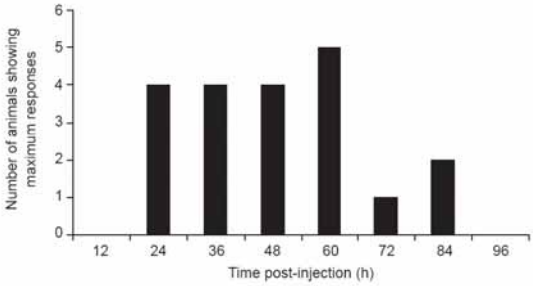


**Figure 2. Number of Iberian red deer hinds (total n=20) showing maximum increase in skin-fold thickness at different times after intradermal injection of 250 µg phytohaemagglutinin in the neck.**

**Table 1. Spearman rank correlations between liveweight and increase in skin-fold thickness in 20 Iberian red deer hinds injected intradermally with 250 µg of phytohaemagglutinin, 12 to 96 h post-injection.**

| Time (h) | Spearman r | P-value |
|---|---|---|
| 12 | 0.056 | 0.812 |
| 24 | 0.559 | 0.010 |
| 36 | 0.484 | 0.030 |
| 48 | 0.585 | 0.006 |
| 60 | 0.545 | 0.012 |
| 72 | 0.565 | 0.009 |
| 84 | 0.436 | 0.054 |
| 96 | 0.349 | 0.131 |

Histopathologically, the skin biopsy showed a diffuse inflammatory reaction with abundant fibroblasts throughout and more intense reaction around blood vessels. The mixed inflammatory infiltrate contained eosinophils, lymphocytes, and some macrophages.

## Discussion

In this study, we demonstrated a dose-dependent response *in vivo* in Iberian red deer hinds to PHA injected intradermally, and that variation over time after injection required the establishment of a

fixed time for measurement of the cellular response. We found that using a fixed protocol and the same observer for the measurement of skin-fold thickness provided reasonable reliability (ICC>0.95). Future research could be directed at determining whether differences in reliability might also depend on the sex, age, form of immobilisation, or condition of the individuals tested.

In pigs, skin responses to PHA were dependent on dose but the kinetics were similar for all concentrations, and the optimum dose was found to be 100 µg (Ekkel et al 1995). Doses of 250 µg PHA were used in cattle (Hernández et al 2005). Our results in deer showed that a dose of 250 µg gave clear and long-lasting responses. Lower doses, while being less expensive, are not recommended according to our results.

Cows had the greatest response 6–48 h (Hernández et al 2005) or at 12 h (Kelley et al 1982) post-injection. Our results showed a delayed and relatively homogenous response between 48 and 84 h post-injection. Hence, a time of 72 h is proposed for PHA skin tests in deer, since tuberculin skin tests are also read at 72 h post-injection (Waters et al 2004), and both techniques would most likely be used concurrently, particularly under field conditions. The inter-specific differences in PHA kinetics suggest that different protocols may be needed for other mammalian taxa.

Ekkel et al (1995) concluded that frequent measurements were preferable to single measurements when the effects of stress on immunological responses were studied. While our data confirmed the existence of differences between animals, they also supported the establishment of an ideal time for measuring change in skin-fold thickness, which would reduce the stress and risks of handling, especially of wild animals (Smits et al 1999); this is often the only compromise between study design and logistical capacity.

Acute stress enhances, while chronic stress suppresses, skin immunity (Dhabhar 2000). Studies using PHA *in vitro* in New Zealand identified a dramatic reduction in the leukocyte blastogenesis of peripheral blood cells from deer in the 4 weeks following capture. The mitogenic reactivity of lymphocytes was suppressed by 90% in the first week post-capture, but recovered so that only 30% suppression was evident 1 month post-capture (Griffin 1989). In our study, animals were used to handling, and all were exposed to the same level of stress. However, care must be taken when comparing the PHA responsiveness of animals under very different conditions.

In cattle, eosinophils predominate at PHA injection sites in the skin, and neutrophil and/or macrophage but not lymphocyte scores were greater in PHA-injected than control animals (Hernández et al 2005). An abundance of eosinophils was also observed in a biopsy from the one deer sampled in this study.

Measurement of immune reactivity is an important tool for defining how animals cope with environmental demands (Hessing et al 1995). Our finding that the response to PHA correlated with liveweight agrees with the work by Sams et al (1996) in white-tailed deer. Liveweight is considered a general indicator of nutritional condition in deer (Loison and Langvatn 1998). In our opinion, this suggests that the *in vivo* response to PHA may prove useful in studies of the ecology of deer, provided the animals can be handled twice within the prescribed time-frame. The use of PHA as a positive control in tuberculin skin testing of farmed red deer could also improve the reliability of that technique, but this requires further research.

## Acknowledgements

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## Efecto del sexo y la edad en el incremento de pliegue cutáneo en la intradermorreacción con PHA en el ciervo ibérico


### Resumen

El objetivo de este trabajo es determinar si en el ciervo ibérico (*Cervus elaphus hispanicus*) existen diferencias relacionadas con el sexo y la edad en la respuesta a la inyección intradérmica de un mitógeno, fitohemaglutinina (PHA).

Para ello se testaron 110 ciervos ibéricos de granja (51 machos y 59 hembras) inyectándoles 250 μg de PHA intradérmicamente en el lado derecho del cuello. La edad de estos animales oscilaba entre 21 meses y más de 5 años. El incremento de pliegue cutáneo se midió a las 72 horas tras la inyección.

Se demostró un efecto significativo del sexo en el incremento del pliegue cutáneo, de forma que los machos tendían a tener una mayor respuesta que las hembras ($p=0.02$). En cambio, no hubo diferencias significativas en relación con la edad del animal.

Los valores de referencia propuestos permiten, en cuanto a dosis y tiempo de lectura, la incorporación del mitógeno a la rutina de las pruebas de intradermorreacción con antígenos micobacterianos. El sexo, probablemente debido a las características ecológicas en el ciervo, debe ser tomado en cuenta a la hora de interpretar los resultados de la intradermorreacción, tanto en ecología como en el control sanitario de la tuberculosis. Se debe evaluar el incremento de pliegue relativo al grosor de la piel del animal más que el incremento per sé, ya que la primera es una medida más apropiada de "incremento real". Este hecho puede tener trascendencia en la interpretación de los resultados de la prueba de la tuberculina.

**The effects of sex and age on phytohaemagglutinin skin-testing of deer**

Fernández-de-Mera IG, Vicente V, Höfle U, Rodríguez O, García A, Gortázar C
New Zealand Veterinary Journal. En prensa

**Abstract**

AIM: To determine if there are sex- or age-related differences in the skinfold thickness increase in response to the mitogen phytohaemagglutinin (PHA) in red deer.

METHODS: Two hundred and fifty μg of PHA was injected intradermally of the right side of the neck in 110 (51 males and 59 females) captive Iberian red deer (*Cervus elaphus hispanicus*). The age range of these animals was from 21-month-old to ≥5-year-old. Skinfold thicknesses were measured at 72 h following injection.

RESULTS: There was a significant effect of sex on the increase in skinfold thickness; males tended to have greater increases than females after correcting for other confunding variables ($p=0.02$). No age-related differences were statistically evident.

CONCLUSIONS AND CLINICAL RELEVANCE: Reference values are provided at a dosage and time of measurement that are in accordance with the logistics of performing comparative cervical tuberculin skin testing of red deer. Sex, probably due to life history constraints in red deer, must be taken into account when interpreting skin-test data, both in ecology and in TB control. Skinfold increase relative to the thickness of the skin rather than skinfold increase *per se* should be evaluated as a more appropriate measure of 'true skinfold increase', which would have clinical relevance in the interpretation of tuberculin skin testing.

KEY WORDS: Cellular immunity, *Cervus elaphus*, mammalian immune response, wildlife bioindicators, phytohaemagglutinin skin test

Abbreviations

CI = Confidence intervals

PBS = Phosphate buffered saline

PHA = Phytohaemagglutinin

SD = Standard deviation

SEM = Standard error of the mean

Tb = Tuberculosis

## Introduction

Measurement of immune reactivity is an important tool to define how animals cope with environmental demands (Hessing et al 1995), and is also valuable by complementing diagnostic tests based on the immune response. The phytohaemagglutinin (PHA) skin test is an inexpensive and easy measurement of *in-vivo* cellular immune responses (Smits et al 1999). PHA, a lectin from *Phaseolus vulgaris*, is a mitogen. The skin test comprises injecting PHA intradermally and measuring the change in skin thickness. The immune response is considered to be proportional to the difference in swelling between the site injected with PHA and a control site injected with phosphate buffered saline (PBS), or the increase in swelling before and after injection of PHA (Kelley et al 1982, Ekkel et al 1995, Smits et al 1999, Hernández et al 2005).

In a previous study (Fernández-de-Mera et al 2006), we demonstrated a dose-dependent *in-vivo* response in red deer hinds to PHA injected intradermally, and that variation over time after injection required the establishment of a fixed time for measurement of the cellular response. Using a fixed protocol and the same observer for the measurement of skin thickness (a double measure) in deer provided reasonable reliability. The results showed that a dose of 250 μg gave clear and long-lasting responses, and a time of 72 h was proposed for PHA skin testing of deer, since tuberculin skin tests are also read at 72 h post-injection (Waters et al 2004), and both techniques would most likely be used concurrently, particularly under field conditions, with the purpose of detecting possible false negatives in the tuberculin skin test, since PHA allows detecting anergic animals.
However, it is necessary to determine whether differences in reliability might also depend on the sex and age of the individuals tested. Hence, in this study we used a large sample of farmed Iberian red deer of different age groups and both sexes (51 males and 59 females) to test for the effects of these factors on the skin test response to PHA.

## Materials and methods

### Study animals

A group of 110 Iberian red deer (51 males and 59 females), comprising 45 21-month-old individuals named yearlings (24 males and 21 females), 13 3-year-old adults (11males and 2 females), 19 4-year-old adults (11 males and 8 females), and 33 ≥5-year-old adults (5 males and 28 females), was kept on the Experimental Farm of the University of Castilla - La Mancha, Albacete, Spain. All the study animals were apparently healthy. The farm is

tuberculosis free, as no animals have tested positive to the comparative cervical tuberculin test, clinical cases or post-mortem evidence of the disease. The animals were accustomed to handling and experienced no detectable behavioural stress. They were individually identified with an ear tag and transponder. All deer were kept in open-air enclosures during the course of the study, which was conducted in February 2003. In this month, late winter in Spain, the majority of stags drop their antlers and most of the hinds are pregnant. The deer were immobilised in a hydraulic crush, for injection and measuring skin thickness. At each injection point, hair was eliminated almost completely prior to intradermal injection with an electric shaved (Moser® Avalon 1290, Valencia, España) in two areas of skin on the right side of the neck, measuring 3 x 3 cm. Each animal was held in the crush for less than 5 min. Animals were weighed to the nearest 0.05 kg, 10 g to 300 kg escale (KC 300 S, Mettler-Toledo SAE, Spain). All use of animals in research was approved by Castilla-La Mancha University Animal Ethics Committee, experiment number 11-100044-141628. Handling procedures and sampling frequency were designed to reduce stress and health risks for subjects, according to European (86/609) and Spanish law (RD 223/1988; RD 1021/2005), and current guidelines for ethical use of animals in research (ASAB, 2006).

### PHA skin testing of cell-mediated immunity

Two injection sites were prepared in the right side of the neck. A volume of 0.1 ml was injected intradermally in one site, with 250 μg/ml of PHA (Sigma-Aldrich, Missouri, USA). A volume of 0.1 ml of PBS was injected intradermally in the other site as a control. One-ml syringes fitted with a 25-G 1/2-inch needle were used. Immediately prior to injection, and 72 h after administration, skinfold thicknesses were measured twice, to the nearest 0.1 mm, using a digital calliper (Mitutoyo, Cardiff, UK), by the same person.

### Statistical analysis

To test the effect of sex and age while controlling for bodyweight on increases in skinfold thickness (as a response variable), a generalised linear mixed model was used. Sex and age (four levels: 21-month-old individuals named "yearlings", 3 year old adults, 4 year old adults, and ≥5 year old adults) were explanatory categorical factors (and the two-way interaction), and live bodyweight was included as an explanatory continuous variable. The effect of the skin thickness prior to injection and total body length (to the nearest 0.1 cm) were also controlled for. Finally, we included in the model the skin thickness increased measured at the negative control (PBS point, mm) to control for any possible increase as a

consequence of mechanical irritation. The reliability of double measures has been previously assessed by Fernández-de-Mera et al (2006). We represent the residuals of the regression of skinfold increase on body weight across sex by age classes to visualize the skinfold increase once corrected by the initial skinfold thickness. The residuals is the difference (or left over) between the observed value of the variable and the value suggested by the regression model, and provides a measure of the relative deviation (positive or negative, which is interpreted in relative terms, but does not resemble a real response) of the dependent variable (skinfold thickness increase) respect to explanatory factors (initial skinfold thickness). We used the standardized residuals (the residual divided by the standard error). Statistical uncertainty was expressed through 95% confidence intervals (CI) of the standard error of the mean (SEM). We used SAS 9.0 Statistical package.

## Results

Figure 1 shows the increase in skinfold thickness in response to PHA as a function of sex and age, including group sizes. No individual deer had aberrant reactions, or lack of a reaction to the test. There were statistical differences concerning sex ($F=4.75$, $p=0.03$, $R^2$ for the model = 0.41); males tended to have greater skinfold increases (8.8 (SEM 0.57, range 0.85-15.3) mm) than females (4.23 (SEM 0.39, range 0.5-9.35) mm) after correcting for other confusing variables.

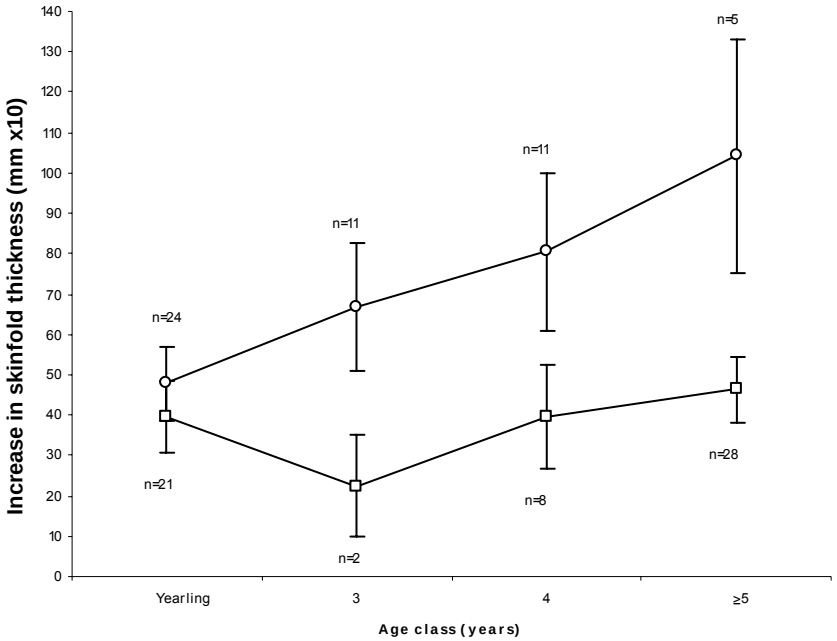


**Figure 1.** The increase in skinfold thickness (± 95% confidence intervals) in the neck of Iberian red deer 72 h after injection of 250 μg of phytohaemagglutinin, in relation to sex (○=males; □=females) and age class.

Figure 2 shows the average standardised residuals of the regression of skinfold increase on bodyweight across sex by age classes. No age-related differences were statistically evident (F=0.95, p=0.41), but differences between sexes were more marked with increasing age (significant sex-by-age interaction: F=2.62, p=0.05; Figures 1 and 2). Skinfold increase after injection of PHA was not related statistically to the initial skin thickness (F=1.33, p=0.25), PBS skinfold increase (F=0.37, p=0.54), bodyweight (F=1.06, p=0.31), or body length (F=2.24, p=0.13).

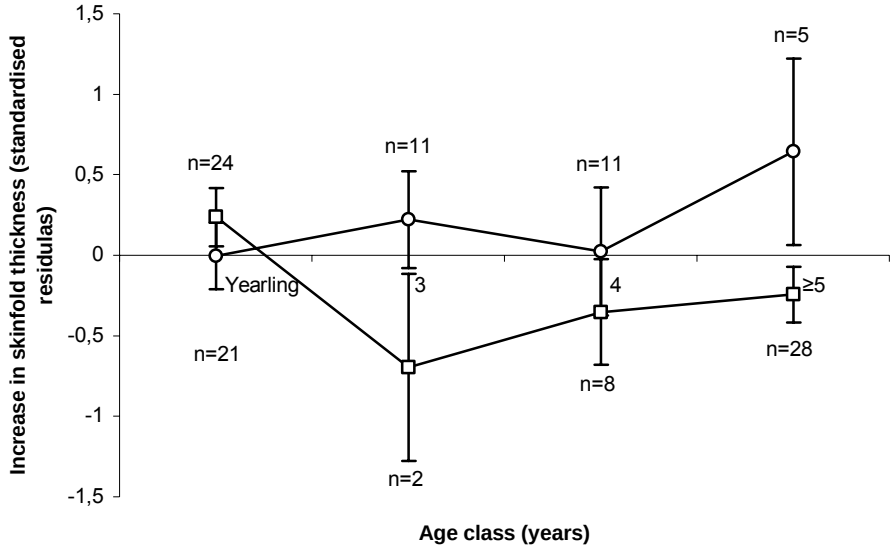


**Figure 2.** The average standardised residuals of the regression of the increase in skinfold thickness on bodyweight across sex-by-age classes of Iberian red deer, 72 h after injection of 250 μg of phytohaemagglutinin, in relation to sex (○=males; □=females) and age class.

## Discussion

In this study, sex-by-age variations in the increase of the skinfold thickness after intradermal injection of the mitogen PHA was described for the first time in red deer. Reference values at a dosage and time of measurement are provided that previously were tested and that are in accordance with the logistics of performing comparative cervical tuberculin skin testing of red deer. The reported skinfold increases of individuals that appear to be in generally good health are useful baseline data for development of a complementary diagnostic tool for specific tests against diseases such as Tb.

The use of PHA along with comparative cervical tuberculin skin testing may prove of special interest in the detection of anergic individuals and therefore could improve the reliability of *in-vivo* diagnostic tests for Tb in red deer. Animals that are immunosuppressed

and therefore non-responsive (or less responsive) to PHA (non specific response) may be unable to also elicit a specific response to Tb antigens. This may encourage future research as the volume of red deer translocated in Spain is high, and undiagnosed Tb can produce uncontrollable spread of the disease to new areas (Vicente el al 2006).

Tuberculosis is an important disease in red deer both in Spain (Vicente et al 2007a) as well as many other countries where red deer are farmed (de Lisle et al 2001). In Spain, testing wild Iberian red deer from Tb endemic areas evidenced a correlation between the skin test responses to PHA and to bovine tuberculin (the authors, unpublished data). Our results suggest that the response to tuberculins could vary as the response to PHA does according to the immunological status of the deer, and more research is needed on this subject. Additionally, the PHA test could be useful in the future for monitoring the general health of red deer populations, and for ecological studies of quantification of the immune response. To the best of our knowledge, no adverse effects of PHA on mammals have been reported, and in our experience there is no interaction between PHA and tuberculin antigens during skin testing of deer.

Males had greater skinfold increases than females (after controlling for the effects of weight, body size and skin thickness), thus when comparing the PHA test with reference values it should take into account the effect of sex. Cellular immune responses may differ between males and females in red deer because reproductive effort and reproductive expectancies differ between sexes (Clutton-Brock et al 1982; Vicente et al 2007b).

Our study was conducted in February, at which time the males were recovering after the strenuous rutting period (September-October), an energetically demanding time (Johns et al 1984) even for animals in captivity. Therefore, they probably recently gained immune function capacity. In the case of the females, they were gestating, which is also an energetically costly period (Clutton-Brock et al 1982), which can be reflected in lower activation of immune function as resources are allocated to reproduction. The fact that gestation is especially costly in primiparous hinds (Landete-Castillejos et al 2004) could explain the decrease evidenced in this group (Figure 2) and the significant sex-by-age interaction, supporting the hypothesis that early reproduction investment in young hinds which are still growing may negatively affect cell immune responses. These results are in agreement with recent data on age by sex variation of spleen size (an indirect measure of immune capacity, Corbin et al 2007) in wild Iberian red deer (Vicente et al 2007b). The apparent absence of sex differences in non-reproductive individuals (calves) is also consistent with this hypothesis. Nevertheless caution must be taken because of the low

sample size. Future research is needed in order to determine seasonal differences in PHA testing of red deer, which may vary across sexes.

We conclude remarking that sex and age should be taken into account when measuring PHA skin test responsiveness in deer. Our findings also suggest that skinfold increase relative to the thickness of skin rather than skinfold increase *per se* should be considered a more appropriate measure of 'true skinfold increase'. These facts could also apply for the interpretation of skin reactions to injection of tuberculins. Future research should focus on this issue, from immunological, biometrical and histopathological aspects.

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## Inyección intradérmica de PPDb, PPDa y PHA en el ciervo ibérico: Factores que afectan a la respuesta de la tuberculina


### Resumen

La prueba de intradermorreacción con derivados purificados de proteínas micobacterianas (PPDs) es hoy día el test estándar para el diagnóstico de tuberculosis en animales. Sin embargo, no existen datos disponibles para el caso del ciervo en España, donde las condiciones de manejo son muy variables, variando desde sistemas de granjas cinegéticas a condiciones silvestres. La intradermorreacción con fitohemaglutinina (PHA) es una medida in vivo de respuesta inmune celular que puede utilizarse combinándola con la tuberculina, con el fin, por ejemplo, de identificar y descartar los individuos anérgicos. El objetivo de este estudio es analizar el efecto del sistema de manejo sobre la capacidad de respuesta del ciervo a la intradermorreacción con antígenos micobacterianos (PPD bovina y PPD aviar) y no micobacterianos (PHA). Se observaron diferencias estadísticamente significativas en la respuesta a la inyección intradérmica de PHA y PPDs entre los ciervos de granja y los silvestres, mostrando los primeros un mayor incremento de pliegue cutáneo. También, e independientemente de los resultados de la intradermorreacción, la respuesta a la PHA se relacionó de forma positiva a la de las PPDs en los ciervos silvestres, pero no en los de granja. Todo esto sugiere que la diferente condición física producida por un manejo intensivo podría reflejarse en la capacidad de respuesta inmunológica. La presumible peor alimentación en el caso de los ciervos silvestres podría hacer que la capacidad de respuesta inmunitaria frente a los antígenos fuese más dependiente del estado inmunitario (como podría reflejar el incremento de pliegue cutáneo tras la intradermorreacción con PHA). Estos resultados proporcionan una información básica útil para contribuir en el diseño de análisis de TB con una adecuada especificidad y sensibilidad en ciervos con diferentes situaciones de manejo. La PHA ha demostrado ser un antígeno potencialmente útil como control positivo en la prueba de la tuberculina en el ciervo, para establecer así los puntos de corte sobre la respuesta inmunitaria en una determinada población sometida a unas determinadas circunstancias, para detectar posibles individuos anérgicos y para interpretar las reacciones no específicas a la PPD aviar. Además, la respuesta a la PHA podría ser una medida de manejo útil para identificar "poblaciones de riesgo" con vistas a mejorar su condición física y así la inmunocompetencia.

**Factors affecting red deer skin test responsiveness to bovine and avian tuberculin and to phytohaemagglutinin in Spain**

Fernández-de-Mera IG, Vicente J, Höfle U, Ruiz Fons F, Gortázar G

**Abstract**

Skin-testing with purified mycobacterial protein derived antigens (PPDs) is still the standard test for tuberculosis in animals, but no data are available for red deer in Spain, where management conditions are variable, ranging from farming to wild. The phytohaemagglutinin (PHA) skin test is a measurement of in-vivo cellular immune responses that can be used in combination with TB skin testing, for example in order to identify and remove anergic individuals. The aim of this study is to test the effect of management on the responsiveness of red deer to skin testing with mycobacterial (bPPD and aPPD) and non-mycobacterial antigens (PHA). We evidenced a statistical different immune response to PHA and PPD injections between captive and free-living deer, farmed deer showing higher skinfold increases. Also, regardless of skin testing result, PHA positively related to bPPD in wild conditions, but not in farms. We suggest that differences in health condition promoted by intensive management could be reflected in immunological responsiveness. The lower plane of nutrition expected for wild animals could favour that immune response againts PPD was more dependent on immune status (as PHA skinfold increase may reflect). This study provides useful baseline information to help designing proper specificity and sensitivity studies of TB in red deer in different management situations. PHA has proven to be a potential useful positive control antigen for skin-testing in red deer to establish cut-off points for the immune reactivity in a given population under given circumstances, to detect anergic reactors, and to interpret non-specific reactions to PPDa. Also, intradermal reaction to PHA could be a useful management tool for the identification of "risk populations" with view to the amelioration of body condition and thus immunocompetence in these.

**Key words:** Cellular immunity, *Cervus elaphus*, paratuberculosis, phytohaemagglutinin, skin test, tuberculosis.

**Introduction**

Deer farming is a growing activity in Spain. Most farms are devoted to producing red deer (*Cervus elaphus*) for restocking rather than producing venison or velvet. Thus, increasing

numbers of deer are released yearly from the farms to the field (usually to fenced estates) for hunting purposes. In addition, over 10,000 wild deer are captured and translocated between hunting estates yearly in Spain (Soriguer et al. 1998). In this context, testing of deer prior to translocation in order to avoid sanitary risks becomes paramount.

Mycobacterial diseases, mainly bovine tuberculosis (bTB) caused by *Mycobacterium bovis* and closely related mycobacteria of the *Mycobacterium tuberculosis* complex, and paratuberculosis (PTB, Johne's disease) caused by *Mycobacterium avium* subspecies *paratuberculosis*, are among the most important health issues in deer farming (Riemann et al. 1979, Chiodini and Vankruiningen 1983, Cliftonhadley and Wilesmith 1991, Griffin and Buchan 1994, Mackintosh et al. 2004). In Spain, bTB is highly prevalent among wild deer, with 15% of red deer from southern Spain showing bTB compatible lesions at necropsy (Vicente et al. 2006). The prevalence of paratuberculosis among Spanish deer is unknown, but clinical cases have occasionally been reported in wild fallow deer (*Dama dama*) (Marco et al. 2002, Álvarez et al. 2005) and in farmed red deer (the authors, unpublished data).

Skin-testing with purified mycobacterial protein derived antigens (PPDs) is still the standard test for tuberculosis in man and domestic animals (OIE 2002). In farmed red deer, studies carried out in New Zealand established 82-86% sensitivity and a 46-76% specificity of the comparative skin test (Griffin et al. 1991, Corrin et al. 1993, Norden et al. 1996). No data are available for Spain, since TB testing of deer is not yet compulsory and only sporadic information on post mortem analysis and recovery of mycobacteria by culture of reactors and non-reactors is available (Vicente et al. 2003).

The phytohaemagglutinin (PHA) skin test is an inexpensive and easy measurement of in-vivo cellular immune responses (Smits et al. 1999), that can be used in combination with TB skin testing, for example in order to identify and remove anergic individuals (Fernández-de-Mera et al. in press). PHA, a lectin from *Phaseolus vulgaris*, is a mitogen. The skin test comprises injecting PHA intradermally and measuring the change in skin thickness. The immune response is considered to be proportional to the difference in swelling between the site injected with PHA and a control site injected with phosphate buffered saline (PBS), or the increase in swelling before and after injection of PHA (Kelley et al. 1982, Ekkel et al. 1995, Smits et al. 1999, Hernández et al. 2005). In previous studies, we reported the optimal time and dose for studying the skinfold thickness increase in response to the mitogen PHA in red deer, and showed that there was a significant effect of sex but not of age on the increase in skinfold thickness; males tended to have greater

increases than females after correcting for other confounding variables (Fernández-de-Mera et al. 2006 and in press).

The aim of this study is to highlight the effect of management on the responsiveness of red deer to skin testing with mycobacterial and non-mycobacterial antigens. We used the PHA skin test to test the hypothesis that individuals of populations of the same species under different management conditions (captive versus wild; different farms) may have differing immune reactivity. We suggest that using PHA as a positive control may help in the interpretation of between-farm differences in tuberculin responses.

## Material and methods

*Sample size, study sites and characteristics of the deer*

Deer sampled in this study included 1.041 animals from 6 Spanish farms and 111 adult wild deer captured by the National Parks Agency in south central Spain (calves have been excluded from this sample).

**Table 1**. Deer sampled in this study (n = 1.041 animals from 6 Spanish farms, and n = 111 adult wild deer captured by the National Parks Agency in south central Spain). Farmed deer were sampled from 2002 to 2007 and wild deer from 2003 to 2005.

| Site | Type | Sampling period | Deer origin and management | Information on mycobacterial diseases |
|---|---|---|---|---|
| 1 | Farm | November 2005 | Diverse geographic origin, including central Europe. | Two clinical PTB cases confirmed by culture and PCR |
| 2 | Farm | February 2004 | Iberian red deer. Limited introductions from the wild. | One case of sublinical *M. avium avium* confirmed by culture and PCR |
| 3 | Farm | August 2005 | Iberian red deer. Limited introductions from the wild. | *M. bovis* is tested yearly and reactors slaughtered. No diagnosis. |
| 4 | Farm | September 2002 August 2005 | Iberian red deer. Limited introductions from the wild. | Two clinical *M. bovis* cases diagnosed by culture and PCR. |
| 5 | Farm | September 2007 | Diverse geographic origin, including central Europe. | No information available. |
| 6 | Farm | February 2005 | Deer from Scotland introduced for venison production. | Two clinical *M. avium avium* cases diagnosed by culture and PCR. |
| 7 | Wild | June to September 2003 and 2005 | Iberian red deer | TB-compatible lesions highly prevalent and several *M. bovis* cases confirmed by culture and PCR (Vicente et al. 2006). |

Farmed deer were sampled from 2002 to 2007 and wild deer from 2003 to 2005 (Table 1). Deer farms have a semi-intensive management scheme, with pasture-rotation and year-round food supplementation. These farmed deer are used to human presence and are managed at least twice a year, including physical immobilisation for measurement, sampling, and administration of antiparasitic drugs. In contrast, wild deer captured alive by the National Parks Agency are usually handled only twice in their lives: the first time to take them from the capture corral to the quarantine facility (in or close to the natural area), and a second time when they are sold and translocated. No supplementary feeding takes place in the areas managed by the National Parks Agency, and deer densities are estimated between 10 and 30 deer per square kilometre.

*Skin testing and sampling procedure*

All deer were handled twice during the skin testing experiment, at time 0 h and time 72 h (Table 2).

**Table 2.** Time sequence of handling of wild and farmed red deer hinds for tuberculin and PHA skin-testing.

| Time since start of the experiment | | |
|---|---|---|
| 0 h | 0-72 h | 72h |
| -Capture and physical restraint in a crash<br><br>-Weighing<br><br>-Identification: eartagging, ageing by tooth eruption and wear.<br><br>-Measurement of body length and thorax perimeter. Blood, fecal, and ectoparasite sampling.<br><br>-Shaving of four areas of 3x3 cm at the side of the neck.<br><br>-3 times repeat measurement of skinfold thickness at each injection site.<br><br>-Intradermal injection* | Maintenance of the animals in captivity:<br><br>-In paddocks (farm) or in the quarantine facilities (natural fenced areas).<br><br>-Daily observation.<br><br>-Food and water is provided ad libitum. | -Physical restraint in a crash.<br><br>-Identification: eartag number.<br><br>-Second blood, fecal, and ectoparasite sampling.<br><br>-3 times repeated measurement of skinfold thickness in each injection site.<br><br>-Release |

*Intradermal injection of 0.1 ml avian PPD (Purified Protein Derivative, *M. avium*, Cooper-Zeltia, Spain), 0.1 ml bovine PPD (*M. bovis*, Cooper-Zeltia, Spain), 0.1 ml negative control Phosphate Buffered Saline solution (PBS) and 0.1 ml positive control phytohaemagglutinin (PHA, Sigma-Aldrich ref. L-8754 at 2.5 mg/ml, diluted in PBS).

The deer were immobilised by physical restraint in a crash (hydraulic in the farm, mechanic in the field). Each animal was blinded with a piece of cloth adapted to the forehead with two elastic bands in order to reduce stress and handling risks. Time for handling in the crash was less than 10 min. The detailed handling sequence and skin testing procedures are summarized in Table 2. From each animal biometric data, ectoparasites, faecal and blood samples were obtained. A deer with a skinfold increase ≥2mm to *M. bovis* PPD and ≥ skinfold increase to *M. avium* PPD was considered positive bTB reactor (Griffin et al. 1991, Corrin et al. 1993, Norden et al. 1996). All deer with a skinfold increase to *M. avium* PPD 3 mm larger than the one to *M. bovis* PPD were considered positive *M. avium* reactors (Kollias et al. 1982). Deer with skinfold increases of less than 0.5 mm to all 3 antigens were considered anergic animals.

*Statistical Analysis*

We used General Linear Models (GLM) to test the factors affecting PHA, bPPD and aPPD skinfold increases as response variables, respectively. In the PHA model, we included sex, management type (farm or wild), *M. bovis* skin testing (positive or negative as aforementioned), and *M. avium* skin testing (positive or negative as aforementioned) as factors. PBS, aPPD and bPPD skinfold increases were included as covariates, respectively. For bPPD skinfold increase model, the model included sex and management type (farm or wild), whereas PBS, aPPD and PHA skinfold increases were included as covariates, respectively. *M. avium* skin testing (positive or negative as aforementioned) was considered as factor. A similar model was built for aPPD as response variable, but interchanging aPPD and bPPD skinfold as response variable and covariate, respectively. In this model, *M. bovis* skin testing (positive or negative as aforementioned) was considered as factor. Spearman rank order correlations were used to check for correlations between the reactions to different antigens. We used the Chi square test to compare prevalences between groups. We employed SPSS 10.0.6 program (SPSS Inc. 1999).

**Results**

Table 3 shows the mean values of skin tests according to site and sex, whereas Table 4 shows the mean values according to sex and management type, and Figure 1 displays the mean values of skinfold increases against the antigens, PHA and PBS with the 95 % confident interval non-outlier range. In 8 cases, reactions to all three agents were less than

0.5 mm. Six out of these were wild deer (5.4%), and only 2 were farmed ones (<<1%; $Chi^2$=32.7, 1 d.f., p<0.001).

**Table 3.** Mean values of skin tests according to sampling site and sex (m: males, f: females).

| | | | ***bovis* PPD** | | | | ***avium* PPD** | | | | **PHA** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Site** | **Sex** | **n** | **Mean** | **SD** | **Range** | **+ve reat. (n, %)** | **Mean** | **SD** | **Range** | **+ve reat. (n, %)** | **Mean** | **SD** | **Range** |
| 1 | m | 0 | | | | | | | | | | | |
| | f | 48 | 8.8 | 7 | 0-26 | 0 | 36.2 | 18 | 10-79 | 17, 35.4 | 30.3 | 9 | 11-56 |
| 2 | m | 51 | 23.7 | 34 | 0-208 | 18, 35.3 | 26.7 | 32 | 0-167 | 6, 11.8 | 64.5 | 32 | 8-153 |
| | f | 59 | 7.1 | 9 | 0-37 | 1, 1.7 | 15.5 | 16 | 0-71 | 4, 6.8 | 42.2 | 21 | 5-93 |
| 3 | m | 32 | 14.8 | 17 | 0-74 | 6, 18.7 | 23 | 19 | 0-63 | 3, 9.4 | 39 | 20 | 4-82 |
| | f | 478 | 11.2 | 11 | 0-62 | 24, 5.0 | 22.8 | 15 | 0-78 | 25, 5.2 | 61.6 | 27 | 3-304 |
| 4 | m | 0 | | | | | | | | | | | |
| | f | 255 | 11.1 | 15 | 0-102 | 26, 10.2 | 17.9 | 17 | 0-91 | 23, 9.0 | 26.1 | 18 | 0-132 |
| 5 | m | 67 0 | 69.3 | 38 | 0-170 | 52, 77.6 | 37.5 | 35 | 0-148 | 7, 10.5 | 12.9 | 22 | 0-98 |
| 6 | m | 3 | 8 | 12 | 0-22 | 0 | 66.3 | 12 | 53-75 | 3, 100 | 47 | 11 | 39-59 |
| | f | 48 | 5.5 | 11 | 0-56 | 1, 2.1 | 23.3 | 20 | 0-73 | 12, 25 | 43.3 | 20 | 0-85 |
| 7* | m | 58 | 16.2 | 26 | 0-89 | 16, 27.6 | 5.8 | 8 | 0-31 | 1, 1.7 | 25.1 | 18 | 0-77 |
| | f | 53 | 6 | 11 | 0-51 | 5, 9.4 | 4.4 | 6 | 0-34 | 0 | 19.6 | 12 | 1-48 |
| All | m | 153 | 41.7 | 41 | 0-208 | 77, 50.3 | 32 | 31 | 0-167 | 19, 12.4 | 37 | 33 | 0-153 |
| farms | f | 888 | 10.7 | 12 | 0-102 | 52, 5.86 | 21.7 | 16 | 0-91 | 93, 10.5 | 47.1 | 28 | 0-304 |
| Tot | m | 211 | 34.9 | 39 | 0-208 | 93, 44.1 | 25 | 29 | 0-167 | 20, 9.5 | 33.8 | 30 | 0-153 |
| | f | 941 | 10.4 | 12 | 0-102 | 57, 6.1 | 20.8 | 16 | 0-91 | 96, 10.2 | 45.6 | 28 | 0-304 |
| | | 1152 | 14.7 | 22 | 0-208 | 150, 13 | 21.4 | 20 | 0-167 | 116, 10.1 | 43.2 | 29 | 0-304 |

*National Park Agency

**Table 4.** Mean values (± standard error) of skin tests according to management type (farmed versus wild) and sex (m: males, f: females).

| | **bPPD** | | | **aPPD** | | | **PHA** | | | **PBS** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | Tot | M | F | Tot | M | F | Tot | M | F | Tot |
| **Farm** | 41.69 | 10.66 | 15.22 | 32.03 | 21.73 | 23.24 | 37.04 | 47.12 | 45.60 | 6.37 | 2.57 | 3.44 |
| (n=1041) | ±1.54 | ±0.64 | ±0.68 | ±1.49 | ±0.62 | ±0.58 | ±2.27 | ±0.95 | ±0.89 | ±0.71 | ±0.39 | ±0.35 |
| **Wild** | 17.20 | 6.27 | 11.98 | 6.48 | 4.54 | 5.57 | 25.36 | 19.63 | 22.65 | 0.70 | 1.50 | 1.08 |
| (n=111) | ±2.50 | ±2.62 | ±2.07 | ±2.42 | ±2.58 | ±1.79 | ±3.68 | ±3.89 | ±2.69 | ±1.03 | ±1.09 | ±0.76 |
| **Total** | 34.95 | 10.41 | 14.91 | 25.01 | 20.80 | 21.57 | 33.83 | 45.56 | 43.37 | 4.53 | 2.45 | 3.03 |
| (n=1152) | ±1.35 | ±0.64 | ±0.64 | ±1.33 | ±0.63 | ±0.57 | ±1.98 | ±0.95 | ±0.86 | ±0.60 | ±0.37 | ±0.32 |

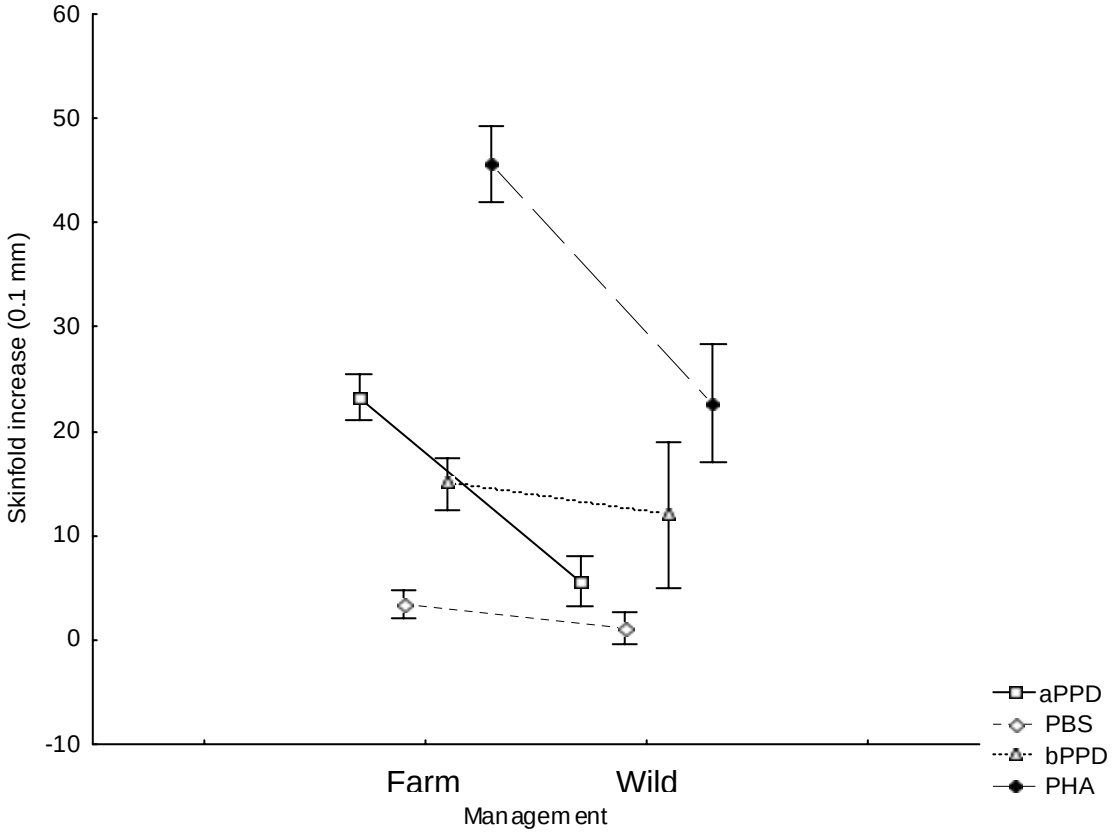


**Figure 1.** Mean increase of skinfold thickness after inoculation of *bovis* PPD, *avium* PPD, PHA and PBS in relation to management. 95 % confident interval non-outlier range is shown.

A total of 93 stags (44.1%) and 57 hinds (6.1%) were considered positive reactors to bovine PPD. The difference in prevalence between sexes was significant ($Chi^2$=220, 1 d.f., $p<0.001$). These positive bovine PPD reactors were identified in 5 of 6 farms and in the wild deer. Prevalence among wild deer (18.9%) was not significantly higher than among farmed deer (14.5%; $Chi^2$=1.5, 1 d.f., $p>0.05$). One farm (site number 5), where only males had been tested, yielded a 77.6% prevalence of positive bovine reactors. Twenty stags (9.5%) and 96 hinds (10.2%) were considered positive reactors to avian PPD ($Chi^2$=6.1, 1 d.f., $p<0.05$). Avian PPD reactors were found among all 7 study populations, but prevalence was significantly lower among wild deer (<1%) than among farmed deer (12.6%; $Chi^2$=13.5, 1 d.f., $p<0.001$). Farm 1, a site with known clinical PTB cases, and farm 6, a site with known clinical avian TB cases, were the two sites with the highest percentage of aPPD skin test reactors.

Results of the GLMs for bPPD, aPPD and PHA skinfold increases, respectively, are shown in Table 5. Figure 2 shows mean least square values for bPPD, aPPD and PHA skinfold increases after the GLMs. Concerning the model fitted for the skinfold increase to bPPD, overall, the effects of management and sex were mediated by their interaction, so

that between-sex differences were evidenced only in farm conditions, whereas wild animals presented similar response across sexes (Figure 2b).

**Table 5.** Test statistics of GLM for skinfold increase of PHA, *bovis* PPD and *avium* PPD, respectively.

| | SS | *F* | Estimate | *p* |
|---|---|---|---|---|
| **PHA** | | | | |
| Management | 1738.6 | 3.90168 | 7.31 | 0.04 |
| Sex | 2254.2 | 5.05867 | 5.31 | 0.02 |
| PBS | 3418.3 | 7.67106 | 0.72 | 0.01 |
| Management*Sex | 1945.3 | 4.36539 | 2.96 | 0.04 |
| Management*bPPD | 1732.9 | 3.88868 | -0.24 | 0.04 |
| **bPPD** | | | | |
| Management | 11504.5 | 38.6662 | 10.17 | <0.01 |
| Sex | 36666.6 | 123.2353 | 12.89 | <0.01 |
| aPPD | 54966.0 | 184.7391 | 0.38 | <0.01 |
| PHA | 1292.4 | 4.3438 | 0.12 | 0.04 |
| Management*Sex | 11236.5 | 37.7654 | 5.83 | <0.01 |
| Management*PHA | 6906.2 | 23.2115 | -0.27 | <0.01 |
| Sex*PHA | 10610.6 | 35.6617 | -0.14 | <0.01 |
| **aPPD** | | | | |
| Management | 3373.1 | 11.6605 | 5.57 | <0.01 |
| bPPD | 53439.9 | 184.7391 | 0.37 | <0.01 |
| Sex | 1498.0 | 5.1786 | -2.74 | 0.02 |
| Sex*PHA | 2690.1 | 9.2997 | 0.07 | <0.01 |

Skinfold increase to bPPD positively related to aPPD and PHA responses, respectively. The relationship with PHA was more marked in females ($r_s$=0.12, n=941, p<0.001) than in males (significant sex by PHA skinfold increase interaction, $r_s$=-0.19, n=211, p<0.01). Also, there exists a statistically positive relationship between bPPD and PHA responses in wild condition, but not in farms (Figure 3, significant management by PHA skinfold increase interaction).

Results of the GLM for aPPD yielded that management statistically affected the aPPD response since animals in farm condition presented higher values (Figure 2c). As previously found, skinfold increase to aPPD positively related to bPPD. The relationship with PHA was more marked in females ($r_s$=0.14, n=941, p<0.001) than in males (significant sex by PHA skinfold increase interaction, $r_s$=0.13, n=211, p=0.05).

Finally, regarding the model on PHA response, sex (higher values in males) and management (higher values under farm conditions) statistically related to the PHA skinfold increase, although both effects were mediated by a significant sex by management

interaction term. This interaction evidenced that between-sex differences existed only in farm conditions, whereas wild animals presented similar response across sexes (Figure 2a).

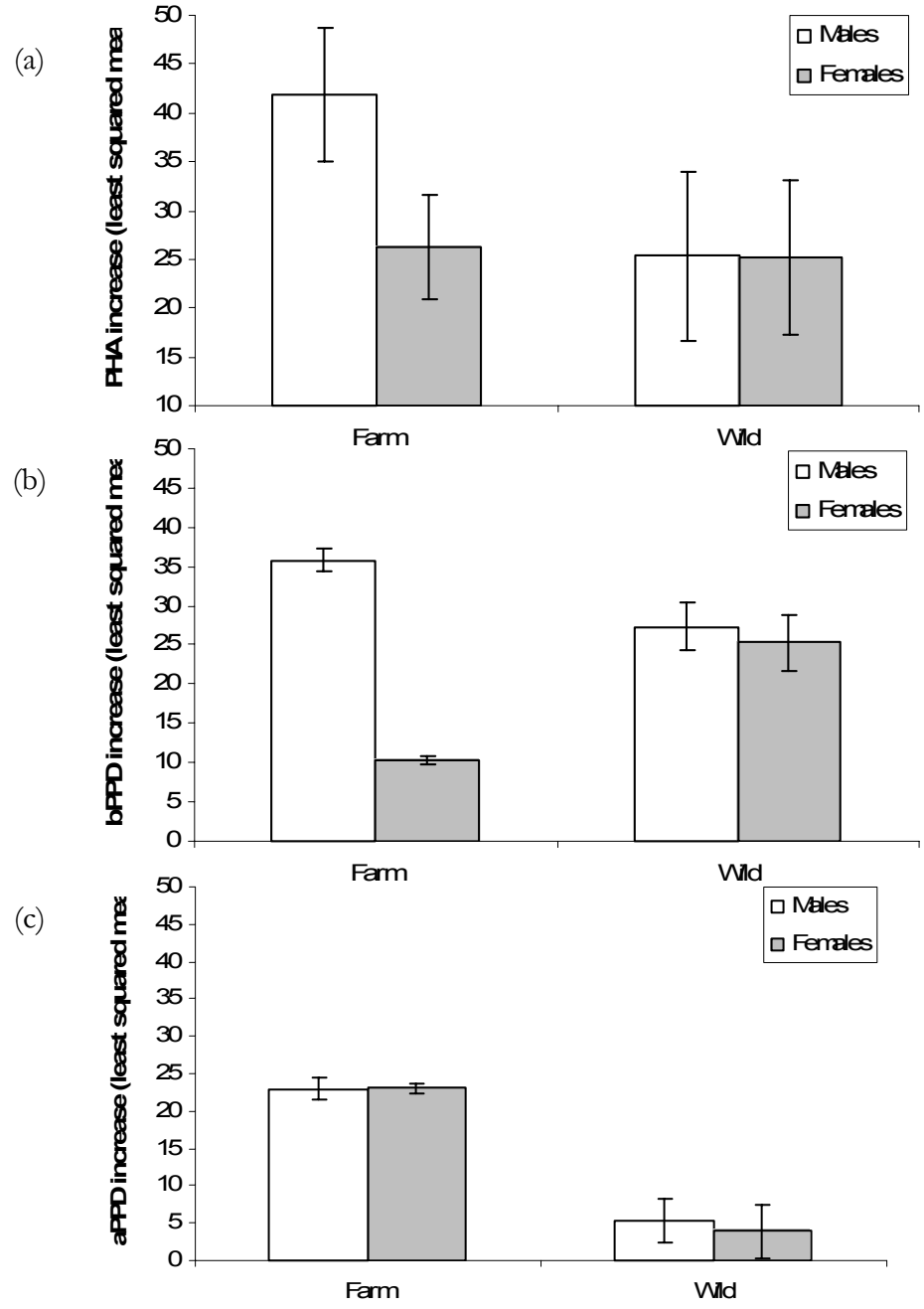


**Figure 2.** Differences between wild and farmed red deer in the increase of skinfold thickness after inoculation of *bovis* PPD, *avium* PPD, and PHA in relation to sex. Values are shown as least square means, and therefore are corrected for other variables of the statistical models.

Management also interacted with the bPPD skinfold increase, and as aforementioned, there was a statistically positive relationship between bPPD and PHA responses in wild deer, but not in farms (Figure 2a, c). The skinfold increase to the injection of the negative control positively related to PHA response. The farm with the

highest proportion of bovine reactors (number 5) showed the lowest response to PHA among all the study sites.

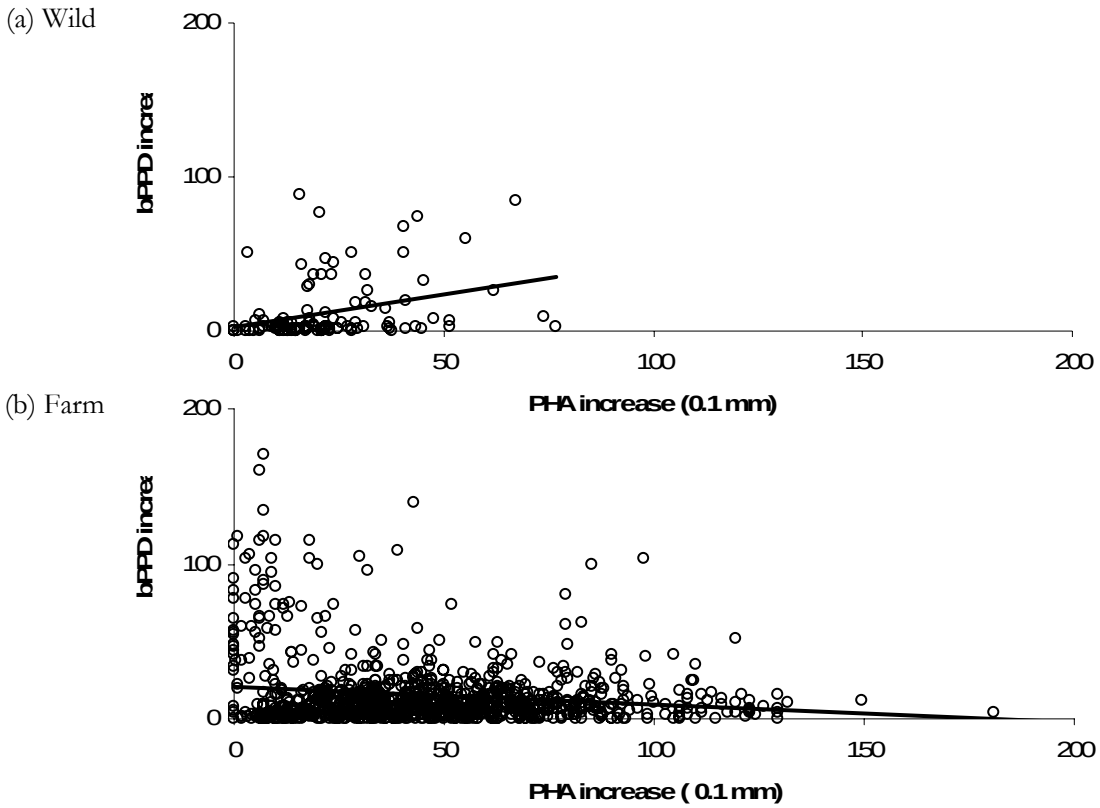


**Figure 3**. Relationship between skinfold thickness after inoculation of PHA and *bovis* PPD in relation to management (a: wild; b: farm)

## Discussion

Different species of wild animals including ungulates such as the red deer have been identified as reservoirs for bovine tuberculosis and are thought to be responsible for the failure of eradication programs in cattle throughout Europe (Caffrey 1994, Hunter 1996). Thus, especially in areas such as central Spain where ungulates are exploited as game species in highly managed environments, tuberculosis skin-testing is becoming an important tool for the management of bTb in these species (Lloydwebb et al. 1995, Griffin et al. 2004, Cousins and Florisson 2005, Palmer et al. 2006).

However, deer skin testing is not yet compulsory throughout Spain, and standardized procedures are urgently needed. This study provides useful baseline information to help designing proper specificity and sensitivity studies. Data presented in this paper belong to different farms and wildlife management situations and were collected opportunistically in different seasons from 2002 to 2007. While the effect of age (less

relevant) and sex (more relevant) on (PHA) skin testing of deer is known (Fernández-de-Mera et al. in press), no information is available on the effect of the season on skin testing. Hence, results need to be interpreted with care as regards the skin test reactor prevalences presented in this study. Nonetheless, the percentage of positive bPPD skin test reactors is within prevalence figures given for TB-compatible lesions at necropsy in Spain (Vicente et al. 2006). Only limited information exists on paratuberculosis in Spanish deer (Marco et al. 2002, Álvarez et al. 2005), and avian tuberculosis has been reported on very few occasions from wild Spanish deer (the authors, unpublished data). Thus, the results of this study suggest that PTB or avian TB may be widespread among Spanish deer farms, a fact that (if confirmed) obliges to set up urgent preventive measures, since most farmed deer are destined to restocking of hunting estates.

The most consistent result in the present study is that deer management (farmed *versus* wild) was identified in all three models as a key factor affecting deer skinfold thickness increase in response both to mycobacterial (bPD and aPPD) and non-mycobacterial antigens (PHA). The differences occurred in the same sense, regardless of some interactions, farmed deer showing higher values (Figures 1 and 2). These findings may relate to two groups of factors. Firstly, differences in general health condition could be reflected in immunological responsiveness. It is of general knowledge that different factors such as nutritional condition and stress may affect immune capacity in mammals (Moller et al. 1998, Coop and Kyriazakis, 1999, 2001, Lochmiller and Deeremberg 2000). For example, wild birds (zebra finches) had lower PHA responses than their aviary-bred counter-parts possibly because energetic costs limited a general resistance response (Ewenson et al. 2001). Body condition is closely related to the T-cell mediated immune response as measured by the PHA skin test in birds (e. g. Soler et al. 2003) which is consistent with our findings in our mammalian model species (Fernández-de-Mera et al. 2006). This difference in immune reactivity may also reflect differences in immunocompetence and in host resistance to diseases in general (Hawley et al. 2007). Stress is also a strong immunsupressor and may alter cellular immune reaction, especially in wild animals such as red deer when submitted to the stress of handling, thus rendering the skin-testing less reliable (e. g. Tella et al. 2001). In this context a standardized positive control would help to correct results of PPD-skin-testing for the immune capacitiy of a particular population.

The second group of factors may relate to differences in the prevalence of mycobacterial infections (or previous contact), and the subsequent effects on the skin

responsiveness against PPDs. It is well known the use and sensitivity of PPD to mycobacterial infections (e. g. Kollias et al. 1982). Also, one possibility is that mycobacterial infections (or previous contact) could activate the immune system (especially the cell mediated response) and therefore promote an increase in the responsiveness against inespecific agents such as mitogen PHA. We did not evidence any effect of skin testing positivity (neither *M. avium* nor *M. bovis*) on the PHA response, which suggests that immune status (measured as PHA response) would determine the responsiveness against PPDs than vice versa. Therefore, the statistical associations between responses to different antigens (especially bPPD, and non-mycobacterial PHA) strongly support that skinfold increase to response to mycobacterial antigen may be well affected by the immunological status of the hosts.

Interestingly, we found that management interacted with the bPPD skinfold increase so that there was a statistically positive relationship between bPPD and PHA responses in wild conditions, but not in farms (Figure). This relationship, and particularly in wild condition, is expected to occur if immune system function of the animals is limited by resource availability (Hines et al. 2007). This is so because in the wild it is more difficult for the immune system to reach such a good level that the variations in PHA response would become not representative of real host immune availability. In the case of farm conditions, on average, animals displayed a high PHA skinfold increase, which could indicate a good condition of the immune system, and probably, a good body condition. Therefore, we hypothesize that bPPD would be less determined by (or related to) the immune system status once the animals are in a high plane of immune capacity.

Finally, a third group of factors underlying the differences between farm and wild animals could relate to the red deer life-history (Vicente et al. 2007). It was very relevant the effect of sex and its interactions in determining the responses to different antigens, independently of the mycobacteria infection status. Particularly, we found, as previously described by Fernández-de-Mera et al. (in press) that sex differences in skinfold increase of the mitogen PHA between sexes were marked in farm conditions (in favour to males), but not in the wild. As similar pattern for bPPD was found, and this supports the second group of possible explanations. As suggested by Fernández-de-Mera et al. (in press) most of the females were in gestation when sampling, an energetically costly period (Clutton-Brock et al. 1984), which can be reflected in a lower activation of the immune function as resources are allocated to reproduction. We speculate that males in farm condition probably are less affected by resources shortage as males in the wild are during the

strenuous period of rutting, which may relate to a good immunological status compared to wild stags. Nevertheless we must be cautious because sample size in males was low and future studies should account for seasonal effects.

PHA skinfold increase was not affected by the PPD skin test results. This suggests that this measure is highly independent of the mycobacterial infection status of the animals, and underlines its possible use as a positive control of general responsiveness to skin testing. In humans, lack of skin induration to intradermal injection of PPD (PPD anergy) is observed in a subset of patients with active tuberculosis (Delgado et al. 2002). In order to detect anergic individuals, immunocompromised patients are screened not only with tuberculin, but also with *Candida* and mumps antigen (Smirnoff et al. 1998). In domestic animals, the existence of anergic individuals (false negatives in skin-testing) is one of the major limits to the success of bTb erradication programs (Barlow et al. 1997), but to the authors knowledge no alternative antigens are commonly used as controls. We used PHA as a positive control, expecting to identify those animals that, being negative to both PPDs, do also not respond to the non-mycobacterial antigen PHA. This use of PHA in combination with comparative skin tests needs further research, but it is interesting to observe that animals with no response to PHA nor to the PPD were detected, and that the farm with the poorest PHA response is the one with the highest percentage of bPPD reactors.

In this study, parameters for body condition, size and physiology were not assessed. However, it may be expected that the ad-lib fed farmed deer were in better general condition than wild deer captured in summer, during the dry season. The different immune response to PHA and PPD injection between captive and free-living deer showed that the two populations differed significantly in immune reaction to these antigens. This may have implications for the interpretation of skin test results: "False-positive" reactions in tuberculosis skin-testing are frequently observed in relation to infections with environmental mycobacteria (Lloydwebb et al. 1995). But in addition to this source of false positives, deer populations with a good response to the non specific PHA antigen may have more false positive reactors to the mycobacterial PPDs, while undernourished or otherwise immunocompromised deer may have more false negative skin-test reactors than healthy deer populations.

In conclusion, the results of the present study prove that a positive control that reflects the immune reactivity of the tested individual should be imperative in tuberculin skin-testing in each species, even in domestic animals, as reactivity may vary between

populations. It also underlines that skin-testing parameters standardized in domestic animals should not be directly employed in wild animals even of the same species, as handling stress or other factors may alter immune reactivity considerably. In this study PHA has proven to be a useful positive control antigen for skin-testing in our model species, the red deer, that allows a) to establish cut-off points for the immune reactivity in a given population under given circumstances as needed, b) helps to detect anergic reactors suffering from chronic tuberculosis and/or impaired immune function, and c) helps to interprete non-specific reactions to PPDa that may result from exposure of wild animals to unspecific nonpathogenic mycobacteria. Finally, the intradermal reaction to PHA could be a useful management tool for the identification of "risk populations" with view to the amelioration of body condition and thus immunocompetence in these.

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# Capítulo 2

## Relación entre condición nutricional y capacidad de respuesta antiparasitaria


**Excreción fecal de *Elaphostrongylus cervi* en relación con la suplementación experimental de alimento en el ciervo ibérico: ¿Afecta la condición nutricional a la capacidad de respuesta antiparasitaria?**

"Faecal excretion of *Elaphostrongylus cervi* (Nematoda) in relation to experimental supplemental feeding in red deer (*Cervus elaphus*): Does nutritional condition improve anti parasite response?"

**Excreción fecal de *Elaphostrongylus cervi* en relación con la suplementación experimental de alimento en el ciervo ibérico: ¿Afecta la condición nutricional a la capacidad de respuesta antiparasitaria?**

**Resumen**

Los cambios ambientales en la disponibilidad de alimento pueden influir en la capacidad del hospedador para responder frente a los parásitos (es decir, en su capacidad inmunitaria), y por tanto, el nivel de infección podría ser un indicador fiable del estado de la población en relación a la calidad del alimento disponible. En España, *Elaphostrongylus cervi* (Nematoda) está ampliamente distribuido en el ciervo (*Cervus elaphus*), y un número de estudios cada vez mayor sugiere que la excreción individual de este parásito se correlaciona con la condición física del hospedador y con la historia natural de éste. Se utiliza un grupo de ciervas a las que se somete durante 4 años a experimentación en cuanto a disponibilidad de alimento, para evaluar así el efecto sobre la excreción de larvas 1 (L1) de *E. cervi* por medio una monitorización con medidas no invasivas. Las condiciones ambientales de ambos grupos experimentales (dos áreas valladas contiguas de 13.5 ha, bajo condiciones ambientales mediterráneas, con 17 hembras de ciervo ibérico en cada una de ellas) fueron similares en cuanto a hábitat y topografía (es decir, la disponibilidad de recursos alimenticios naturales era similar). Los parámetros fisiológicos (longitud corporal, peso corporal y hematología) y el nivel de excreción de parásitos al inicio del estudio no diferían en ambos grupos. Durante el periodo de estudio se hicieron evidentes diferencias en la excreción de parásitos de ambos grupos, de forma que las hembras sometidas a suplementación de alimento mostraban unos niveles de excreción menores estadísticamente significativos en los años 3 y 4. Principalmente, nuestros resultados indican que la condición nutricional podría jugar un papel importante en la respuesta del hospedador a la infección parasitaria, y las diferencias en la disponibilidad (suplementación) de alimento entre ambos grupos podrían explicar por qué el grupo suplementado tiene una excreción menor. Las modulaciones de la capacidad inmunitaria en respuesta a condiciones nutricionales podrían tener un efecto significativo sobre la excreción larvaria de *E. cervi*, de forma que el nivel de parasitación podría servir como indicador de la condición física de las ciervas. La respuesta de *E. cervi* a la disponibilidad de alimentos, que en última instancia depende de una base inmunológica, podría constituir una herramienta para la monitorización no invasiva de las estrategias de gestión en poblaciones de ciervo.

**"Faecal excretion of *Elaphostrongylus cervi* (Nematoda) in relation to experimental supplemental feeding in red deer (*Cervus elaphus*): Does nutritional condition improve anti- parasite response?"**

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**Abstract**

Environmental changes of food availability may have consequences in the host ability to cope with parasites (i. e. immune capability), and therefore rates of infection maybe reliable descriptors of population status in relation to forage quality. *Elaphostrongylus cervi* (Nematoda) is wide spread in red deer (*Cervus elaphus*) in Spain, and an increasing body of science suggests that individual excretion correlates with host life history and fitness traits (body condition, immune status). We used a 4-years supplemental-feeding experiment in red deer hinds to examine effects of food availability on the evolution of larval (L1) counts of *E. cervi* by means of non-invasive monitoring. The environmental conditions of both experimental groups (two 13.5 ha contiguous enclosures under Mediterranean conditions, 17 females in each) were similar in terms of habitat and topography (i.e. natural food resource availability). Physiological parameters (body size, body weight and haematology) and parasite excretion rates did not differ at the beginning of the study. Across the study period, differences in *E. cervi* L1 excretion became evident, fed hinds showing statistically lower rates in years 3 and 4. Overall, our results indicate that nutrition might have played a role in host response to parasite infection, and the difference in available (supplemented) food between groups could explain why the fed group had decreased parasite counts. Adjustments in immune function in response to foraging conditions may have significant effects on the larval output of *E. cervi*, and thus parasite rates would reflect fitness benefits for fed hinds. The response of *E. cervi* to forage availability, which ultimately relies on an immunological basis, may contribute to monitor management strategies for red deer.

**Keywords:** *Elaphostrongylus cervi*, red deer, supplemental feeding

## Introduction

Parasites cause constant waste on the energetic resources of most free-living organisms by significantly influencing their host's behaviour, demography and evolution. (Minchella and

Scott 1991; Sheldon and Verhulst 1996, Tompkins et al. 2002) Therefore, the ability of the immune system to respond to any challenge is an important fitness-associated trait and it is vital for defence against pathogens in vertebrates. On the other hand, from a management perspective, parasitological indices can be useful as indicators of the population health status (Citterio et al. 2004, Vicente et al. 2007a). In this sense, one of the main factors that is expected to affect the host ability to cope with parasites is the environmental change of food availability. This is so because host individual capacity to deal with parasites requires metabolic resources, which in turn are highly dependent upon external resource availability.

The extent to which an individual partitions resources into immune defence and other functions in relation to individual fitness has been the subject of much research and debate in the last decade. Nevertheless, few "*in situ*" experimental studies have analysed whether forage conditions, one of the ultimate factors, affect host-parasite relationship, and in particular, in ungulate species (De Neve et al. 2007). Assessing the effects of management on host–parasite interactions in wildlife remains as an important research issue which would be very valuable to establish whether and how rates of infection become reliable indicators of population status in relation to management and forage quality in practical situations. Also, this approach specially applies for species in which disease emergence or increased disease risk is also a frequent consequence of intensive management and overabundance, such as many ungulates (Côté et al. 2004; Acevedo et al. 2006; Gortázar et al. 2006; Vicente et al. 2007b).

We selected a parasite system composed of *Elaphostrongylus cervi* Cameron 1931 (Nematoda: Metastrongyloidea) and the red deer (*Cervus elaphus*), the parasite endemically persisting and wide spread in red deer populations from Spain. The development of novel non-invasive survey methods of parasitological indices and the comparison of standard samples among populations would be highly applicable for red deer in Mediterranean habitats, where populations are locally overabundant, as a result of intensive game management (Gortázar et al. 2006). Such management schemes mainly include supplemental feeding, especially during critical summer periods (Vicente et al. 2007a). Also, there exists an increasing and recent body of science which suggests that individual excretion of *E. cervi* larvae correlates with red deer life history, fitness traits (such as body condition and spleen size; Corbin et al. 2007; Vicente et al. 2007a) and management strategies (in terms of food supplying, Vicente et al. 2007b), which would provide consistent elements of discussion to understand underlying factors in a non-invasive experimental design. The selected red deer population, where availability of resources in

two experimental groups was initially similar, was manipulated to test whether parasite host relationship was conditioned by supplemental feeding.

Concerning the selected parasite, previous research infections in cervids suggests that host immune response is able to modulate the establishment and/or larval outputs of extrapulmonary lungworms (Gaudernack et al. 1984; Corbin et al. 2007; Vicente et al. 2007a, b). In particular, *E. cervi* parasitizes the central nervous system and skeletal muscles of red deer (Mason 1995; Lankester 2001). Red deer acquire infection by accidentally ingesting gastropod intermediate host containing infective larvae. They are liberated in the deer gut and mature during migration in the CNS (subarachnoid spaces), and subsequently migrate into the fascia and connective tissue around skeletal muscles where they mature and live in reproductive pairs and groups (Handeland et al. 2000). Presumably, adult females lay eggs which by the haematogenous route, reach the lungs where they hatch as first-stage larvae (L1). These travel up the bronchial tree, are swallowed, and dispersed in the host faeces. In the environment, L1 penetrate the foot of terrestrial gastropods, where they develop to the infective L3 (Rezac et al. 1994). Faecal examination of Protostrongylid larvae extracted from faeces is a common method of diagnosis in wild ungulates (e. g. Festa-Bianchet 1991; Arnett et al. 1993), and can provide a useful index of relative parasite abundance (Todd et al. 1970; Mason 1989) and/or the resolution of host-parasite interaction.

We used a 4-years supplemental-feeding experiment in red deer hinds to examine effects of food availability on the evolution of larval (L1) counts of *E. cervi* by means of non-invasive monitoring. As parasite resistance of individuals is expected to vary as body resources do, we hypothesize that parasite abundance would become lower in the non supplemented group as feeding advantages become evident in the fed group along the study period.

## Material and Methods

*Study area and experimental design*

The selected area was located in a hunting Estate in the province of Ciudad Real at the Guadiana river valley (South Central Spain; 38º55'N; 0º36´E; 600-850 m above sea level, see Figure 1). This Estate has largely been involved in ecological and sanitary studies of Iberian red deer (*Cervus elaphus hispanicus*) for the last decade (more information can be seen in Vicente et al. 2004; Vicente et al. 2005; Landete-Castillejos et al. 2004). The climate is

Mediterranean, and therefore dry summer is a recurring phenomenon usually accompanied by undernutrition in overabundant ungulate populations. Annual rainfall presents considerable variation (300 to 700 mm), and during the study period was 397.4 mm (2003), 636.9 mm (2004), 226.9 mm (2005), 320.3 mm (2006). The rainfalls during April and May were 154.9 mm (2004), 46.0 mm (2005), 76.2 mm (2006) and 208.7 mm (2007). We fenced two contiguous areas (13.5 ha each, Figure 1) mainly composed by *Quercus ilex* and *Cystus ladanifer* (in lower proportion *Quercus coccifera*, *Rosmarinus oficínalis*, *Arbutus unedo*, *Lavandula stoecha* and *Phillyrea angustifolia* as predominant species) scrublands (3.2) and pastures (10.3 ha in each group). The design of the enclosures was assessed by a Digital model of land uses so topographical conditions and orientation were similar. The study area had been kept fenced to prevent from ungulate grazing for more than 10 years before the study started.

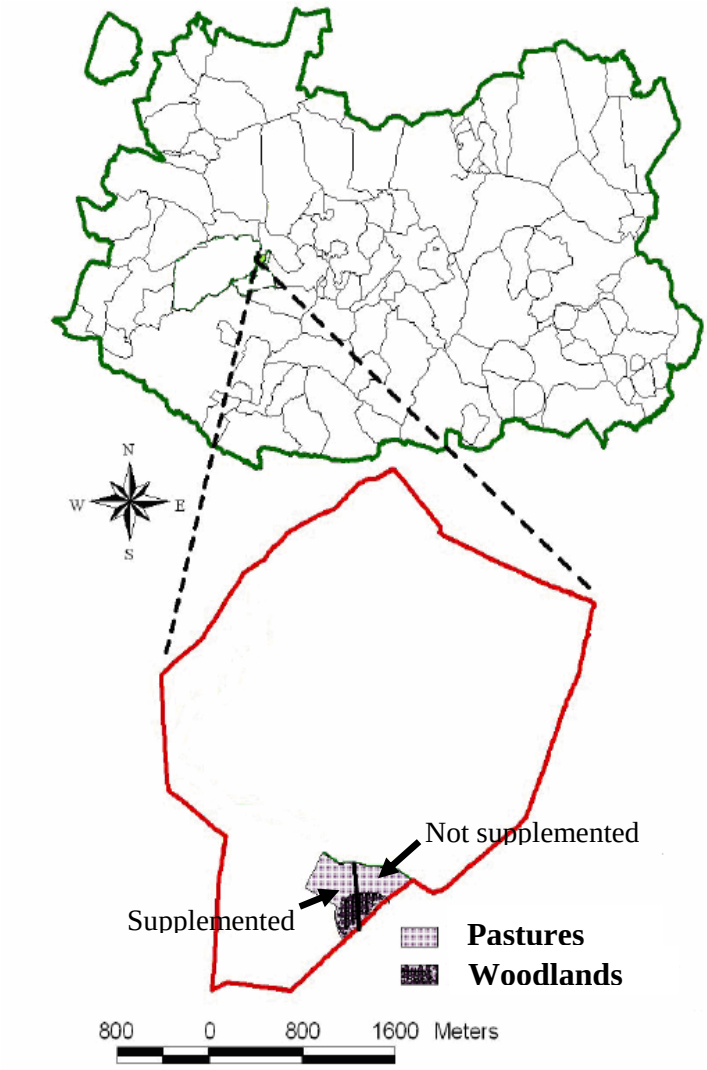


Figure 1. Map of Ciudad Real province (South Central Spain) and detail (below) of the experimental area within the Estate.

In September 2003, 9 female red deer (2 calves and 7 hinds) were introduced in each enclosure. The animals were kept during 3 day in quarantine before they were definitively allocated in a given enclosure, during which individual biometry was performed, and blood and faecal samples were taken to characterize individually parasitological and physiological status. Mean parameters according to experimental group are shown in Table 1.

Table 1. Biometry, physiological parameters and *E. cervi* L1 mean faecal excretion in the individuals introduced in September 2003. Statistics of Mann-Whitney comparisons are shown in the right column. No significant differences between groups were evidenced for any parameter at the beginning of the experiment.

| Parameter | Supplemented (n = 9) | Not supplemented (n = 9) | Mann-Whitney U (*p*-value) |
|---|---|---|---|
| **Live body weight (kg)** | 63.63 ± 5.20 | 65.06 ± 4.19 | 13.5 (0.96) |
| **Body size (cm)** | 153.75 ± 6.09 | 157.16 ± 3.90 | 32 (0.99) |
| **Total serum proteins (mg/dl)** | 8.45 ± 0.15 | 8.69 ± 0.17 | 21.5 (0.38) |
| **Red blood cells (nº/Neubauer camera)** | 249.43 ± 12.31 | 235.71 ± 21.44 | 20.5 (0.34) |
| **Packed red blood cells (%)** | 48.29 ± 1.98 | 46.00 ± 1.56 | 17.0 (0.45) |
| **White blood cells (nº/Neubauer camera)** | 137.88 ± 16.42 | 135.00 ± 13.01 | 26.7 (0.82) |
| ***E. cervi* L1 (nº/g)** | 117.99 ± 51.75 | 102.16 ± 43.67 | 27.2 (0.91) |

In November 2004, 9 female red deer hinds were randomly added to each group since previous management was not possible. All the animals were free-living wild red deer captured using permanent capture enclosures in Cabañeros National Park (50 km far) and were immediately transported to the Estate facilities.

No supplementary feeding takes place in one of the group in the fenced area, these animals only had the natural forage, whereas to the other group nutritional supplement rich in protein was offered to them *ad libitum*.

*Host sampling and laboratory analysis*

We studied fortnightly periods comprised between April 2004 and May 2007, and therefore, this time included 4 annual periods. We visited the experimental enclosures every 15 days to collect fresh fecal pellet groups (those with fresh mucus covering the pellets) from 2 fixed transects within each enclosure. Small sized faecal group pellets coming from calves were not collected. We avoided collecting samples in rainy days since *E. cervi* L1 may

early migrate from faeces, thus the day of sampling varied slightly between months. Sampling in the selected months of the year had the advantage of standardising seasonal variation in parasite prevalence or intensity of excretion (Vicente et al. 2005). Sampling size per experimental group and season is shown in Table 1.

Protostrongylid larvae were extracted in less than 24 h from 8-10 g of faces collected using the Baermann beaker extraction method as described by Forrester and Lankester (1997). Larvae were quantified in a Favatti counting chamber and expressed as number of L1 per gram of faeces. Microscopic measurements were made with a calibrated ocular micrometer and the larvae were identified to the genus level according to their morphology and linear dimensions using the descriptions in Kutzer and Prosl (1975), English et al. (1985), Demiaszkiewicz (1986), Rezac (1990), Mason (1995), Lankester (2001), Vicente and Gortazar (2001). Results are expressed as number of L1 per gram of faeces

*Statistical analysis*

We conducted analyses for the factors affecting parasite counts (faecal *E. cervi* L1 abundance) by means of a generalized linear model (Crawley 1993). The model included individual faecal group *E. cervi* L1 counts (log-10 transformed) as a response variable. We included the variables the type of group (control or not fed *vs.* experimental or food supplemented) and year (from 2003 to 2007) as categorical explanatory factors. We considered a Poisson error and an identity link function (Wilson and Grenfell 1997). We controlled for overdispersion. The resulting saturated up to two interactions models was reduced to their simplest form by eliminating in a backward stepwise manner any explanatory variables or interactions that failed to explain significant variation in the response. Non-parametric Mann-Whitney test were used to compare parasitic, biometry and physiological parameters between experimental groups at the beginning of the experiment. The level of significance was established at the 5 % level. Statistical parameters and phenotypic traits in the text are expressed with SE.

## Results

No significant differences between groups were evidenced for any parameter at the beginning of the experiment (Table 1). Prevalence for the total sample was ± % (*n* = ) and mean abundance was ± % *E. cervi* L1/g. Figure 2 displays prevalence and mean

abundance according to experimental group through the study period. There were statistical differences between groups (Wald = 9.33, $p$ = 0.02) both groups showing an increase of *E. cervi* L1 excretion up to 2006, and a marked decrease in 2007. Whereas the experimental group itself was not statistically significant (Wald = 2.39, $p$ = 0.12), we found that the interaction between group and year was (Wald = 8.51, $p$ = 0.03), so that the fed group showed lower parasite excretion rates from 2006 onwards (Figure 2).

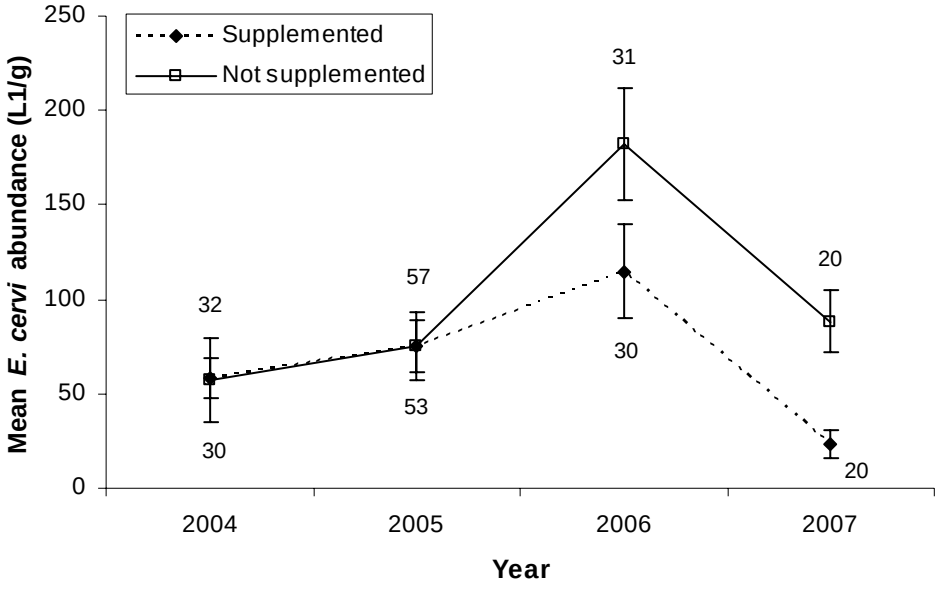


Figure 2. Evolution of the mean abundance of *E. cervi* L1 faecal excretion according to experimental group (intervals represent standard errors). Sampling size for not supplemented (above) and not supplemented (below) groups is shown.

## Discussion

Only a few experimental studies have experimentally shown that artificial supplementation of food reduces vulnerability to parasites. This research provides an example of how environmental factors underlie host-parasite relationships. As we will discuss, the general mechanism proposed for these relationships is an environment resource-dependent immune system hypothesis, in which individuals with a higher availability of resources are in better immunological status.

Numerous factors that can influence and/or mask host-parasite interactions. One key point we must state is that our experimental design, regardless of the factor "supplemental feeding", allowed us controlling any environmental factor that could differentially affect the excretion profile of *E. cervi* between experimental groups. Habitat, topography, climate and host density were identical in both experimental contiguous areas. Therefore, we can assume that our results reflect differences due to the factor

"supplementary feeding". Nonetheless, the main faw of our study design resides in the absence of a second replica, so we can qualify our study as a pseudo-experimental approach. From our point of view, even in the absence of a replica, our study resembled "in situ" conditions and similar infection figures of naturally infected individuals, which is exceptional in the literature. This is very valuable, since it provides a situation with direct applications to practical management of red deer populations (Hines et al. 2007). In addition to its practical value, our paper adds understanding to ecological aspects of the host-parasite relationships and the immuno-epidemiology of wildlife populations.

Previous research in the study area has evidenced that at the individual level, *E. cervi* L1 counts and body condition associates negatively (Vicente et al. 2007a, b), which is what one would expect considering that individuals in prime body condition would invest more (or more efficiently) in antiparasitic defences (Moller et al. 1998; Lochmiller and Deeremberg 2000). In Mediterranean habitats from SCS (which is applicable to other situation of ungulate management world-wide), high deer population densities are maintained by artificially increasing the carrying capacity with supplemental feeding (Vicente et al. 2007a). The consequence is that there is a disproportionally better condition in animals from more intensively managed populations (more food provided), and a subsequent improved resistance against *E. cervi*, or at least, reduced faecal larval excretion. These findings support our environment resource-dependent immune system hypothesis, in which individuals with a higher availability of resources are in better immunological status.

It is well known that the nutritional status of the host can influence the rate of acquisition of immunity to parasitic and other infections in man and many animal species, including ruminants (e. g. Coop and Kyriazakis 1999; 2001). Low levels of dietary protein have been associated with increased helminth faecal egg counts in both sheep and goats (Theodoropoulos et al. 1998; Chartier et al. 2000), and low resource availability due to climate drought also may increase faecal counts in wild ungulates (Ezenwa 2004). Host nutrition (and subsequent improved body condition) can increase the ability of the host to cope with the adverse consequences of parasitism, and also affects the development of the host-immune response and the establishment and survival of parasite populations (Strain and Stear 2001). The partitioning of available nutrients between immune responses and other body functions could in part explain the immune unresponsiveness to *E. cervi* infection and subsequent larval outputs since host immune response modulates the establishment and fecundity of parasites (Gaudernack et al. 1984). Wild ungulates under

drought conditions, like this seasonally prevalent in Mediterranean habitats, may be unable to maintain adequate nutrition, and subsequently be less able to cope with parasite infections Ezenwa 2004). Reduced nutrient intake, like proteins, and dietary deficiencies may associate with declining resilience and resistance to infection. In our Mediterranean environment, a limited availability of quality food in the non supplemented group along the study period compared with the control group could relate to the exacerbated parasite excretion rates in the latter. In fact, such densities of red deer in our study area may cause to damages to the Mediterranean vegetation (the authors, personal observations).

Animals often face a trade-off between investment on anti-parasite defences and other activities related to self-maintenance, survival and reproduction (Zuk and Stoehr 2002; Moller and Saino 2004). As food resources declined in the control group, nutrient/protein deficient animals could have experienced a breakdown in immune function (Coop and Kyriazakis 1999). For example, immune response against gastrointestinal parasites in ruminants causes reductions in worm fecundity, resistance to larval establishment and expulsion of adult nematodes (Balic et al. 2000; Claerebout and Vercruysse 2000), and all three of these factors affect host faecal output. There is some evidence that there is an interaction with nutrition such that animals in good condition can afford to invest more in immunity and hence will have large spleens (Møller et al. 1998), and this, particularly, has been suggested for red deer in relation to *E. cervi* infection (Vicente et al. 2007a; Corbin et al. 2007). It also has been found that a positive relationship between body condition and spleen size occurs in red deer (Vicente et al. 2007a). These precedents indicate a greater investment in immune defence by animals of high body condition since immune defence is costly to mount and maintain (Møller et al. 1998; Lochmiller and Deeremberg 2000). Individuals in prime body condition would invest more (or more efficiently) in antiparasite defences and parasite infection levels would be determined by the immune system.). Therefore, *E. cervi* L1 outputs may reflect higher investment in immunity by individuals in better condition as the relative cost (resource mobilization relative to resource availability) for them is lower (Møller et al. 1998).

As alternative explanation to our results, control red deer may have been exposed to more parasites if there was a higher density of infective intermediate hosts on the enclosure, or if ingestion of intermediate hosts increased. These are unlikely explanations of the results because both enclosures were contiguous and exposition to parasite intermediate forms should be similar. In addition, after an initial infection, meningeal worms are long lived parasites (with life-span similar to those of the hosts, Watson 1984),

and parasite outputs during life is more dependent on the host-parasite relationship than on the rate of parasite acquisition and reinfection.

We found inter-annual variation in *E. cervi* L1 abundance regardless of the experimental group. This could relate to age-profile of infection as animals got older through the study period. The majority of macroparasite systems show age-intensity curves with an initial increase after the age at which an animal is first susceptible to infection (Wilson et al. 2001). Mechanisms which may generate different age-infection profiles in the hosts include parasite-induced mortality, age-dependent changes in exposure to parasites, acquired immunity and age-related changes in predisposition to infection (Hudson and Dobson 1995; Wilson et al. 2001). Epidemiological patterns suggestive of acquired immunity have been found in red deer in our study area (Vicente et al. 2006), in the white tailed deer against reinfection with *Paralephastrongylus andersoni* (Prestwood and Nettles 1977) and *Paralephastrongylus tenuis* (Slomke et al. 1995), the reindeer/*E. rangiferi* system (Gaudernack et al. 1984) and the caribou/*E. rangiferi* system (Ball et al. 2001). Also, inter-annual variation may associate to climatic variations (Vicente et al. 2004), although this seems unlikely as the annual precedent rainfall, and also the April to May rainfall patterns during the study periods were different to mean annual parasite rates (see rainfalls in Material and Methods section to compare with Figure 2).

Although host–parasite interactions are increasingly being considered in the management of wildlife species (Gortazar et al. 2006), very little about the impact of management on parasitism and infectious diseases in red deer is still known. In conclusion, this research provided vital base line information for management of overabundant populations of red deer through its distribution area. We based our research on a parasite species for which an increasing body of science has been developed during recent years (e. g. Corbin et al. 2007; Vicente et al. 2007 a, b, c), which allowed us discussing our non-invasive study in terms of individual relationships of immune status, body condition and parasite rates. Concerning the generalization of our results to other macroparasite infections, the response to host densities, the mechanisms of host immune response against different helminths to reduce their establishment, survivorship and fecundity are similar in part (e. g. Paterson and Viney 2002; Maizels et al. 2004), which suggests that the described pattern for *E. cervi* could present similarities to other low pathogenic helminths. Nevertheless, the relative weight of exposition to parasite and reduced susceptibility due to the effects of supplementary food may be different for parasites of direct transmission which may be more favoured by host aggregation at feeders than those of indirect cycle.

Hines et al. (2007) have reported that supplementally fed elk (*Cervus elaphus nelsoni*) had significantly higher gastrointestinal nematode egg counts than unfed elk in January and February, but significantly lower counts in April, which suggest that supplemental feeding may both increase exposure and decrease susceptibility of elk to gastrointestinal nematodes, resulting in differences in temporal patterns of egg shedding between fed and unfed elk. Finally, we suggest that well-distributed parasitic species across the distribution range of their hosts are good candidates to be used as parasitic indices of health condition (for example, helminths are consistently present in wild ruminant populations worldwide and have in general a subclinical impact). Their complementary use would improve our understanding since multiple infection are the common feature in wildlife. The study of *E. cervi* infection of red deer at population level and extended in time, together with other ecological indicators, may contribute to monitor and develop management strategies. For this purpose, it is critical understanding how environmental and density dependent factors underlie host-parasite relationships.

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**Ethical statement**

This study has been supported by Castilla-La Mancha Government and complies with the Spanish and Castilla-La Mancha laws of animal experimentation.

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# Capítulo 3

## El factor genético: Polimorfismo del MHC II en ciervo ibérico

**Polimorfismo del MHC II en una población aislada de ciervo ibérico (*Cervus elaphus hispanicus*)**
"Major histocompatibility complex class II polymorphism in a hunter-managed isolated Iberian red deer (*Cervus elaphus hispanicus*) population"

**Relación del complejo mayor de histocompatibilidad de la clase II con infecciones y algunos indicadores de condición en el ciervo ibérico**
"Major histocompatibility complex class II polymorphism in relation to infections and life history traits of management relevance in Iberian red deer"

## Polimorfismo del MHC II en una población aislada de ciervo ibérico


### Resumen

El complejo mayor de histocompatibilidad (MHC) contiene los genes funcionales más variables descritos en vertebrados. Los individuos de poblaciones silvestres tienen constantemente que tratar con una gama diversa de patógenos y el polimorfismo de los loci del MHC es lo que determina la diversidad de los antígenos extraños que puede reconocer el sistema inmunitario del hospedador. El polimorfismo en determinados loci podría conllevar a haplotipos variables del MHC clase II. Así, en poblaciones naturales, existe una asociación entre la variación genética de los loci del MHC y las características de la historia natural de la población. En este trabajo se caracteriza por primera vez la diversidad alélica del segundo exón de la cadena DRB-2 del MHC clase II en ciervo ibérico (*Cervus elaphus hispanicus*). La población analizada fue muestreada en una finca vallada del centro sur de España, sometida a manejos con fines cinegéticos, y podría proporcionar información del efecto de las medidas de gestión cinegética sobre la diversidad genética de esta especie.

Los ciervos presentaron altos niveles de variación en el MHC II DRB-2 con 18 alelos diferentes detectados en los 94 individuos analizados. Sin embargo, el 92.5% de los individuos de esta población aislada pero en régimen de cría en libertad eran homocigotos para DRB-2. La proporción de heterocigotos era estadísticamente diferente entre sexos. El índice de diversidad anual de los alelos del MHC II DRB-2 disminuyó significativamente a lo largo de los 16 años del período del estudio. Se analizó también la diversidad genética de tres microsatélites (como marcadores neutros) para diferenciar así los efectos selectivos y los poblacionales en el MHC II de estos animales. La heterocigosidad esperada fue mayor de 0.5 en los tres microsatélites y todos ellos se encontraban en equilibrio de Hardy-Weinberg.

Se cree que el aporte artificial de alimento y la dispersión limitada por el vallado de la finca podrían dar lugar a una endogamia localizada y a una subdivisión de la población. Estos resultados apoyan la importancia de estudios inmunogenéticos para determinar medidas de manejo, especialmente en poblaciones aisladas de ungulados.

**"Major histocompatibility complex class II polymorphism in a hunter-managed isolated Iberian red deer (*Cervus elaphus hispanicus*) population"**

Fernández-de-Mera IG, Vicente J, Pérez de la Lastra JM, Mangold AJ, Naranjo V, Fierro Y, de la Fuente J, Gortázar C
BMC Ecology. En evaluación

## Abstract

***Background***

The major histocompatibility complex (MHC) contains the most variable functional genes described in vertebrates. Individuals from natural populations deal constantly with a diverse range of pathogens and the polymorphism at MHC loci is what determines the diversity of foreign antigens that the host immune system can recognize. Polymorphism at individual loci may result in variable MHC class II haplotypes. Associations between genetic variation at MHC loci and life history traits of individuals in natural populations exists. We characterized for the first time the allelic diversity at the second exon of the b (DRB-2) chain of the MHC class II locus in the Iberian red deer (*Cervus elaphus hispanicus*). The studied population was sampled from a fenced estate in south-central Spain that has been managed for hunting purposes and may provide information of the effect of game management on the genetic diversity of this species.

***Results***

Deer presented high levels of variation at MHC class II DRB-2 with 18 different alleles detected in 94 individuals analyzed. However, 92.5% of the individuals in this isolated but free breeding population were homozygous for DRB-2. The proportion of heterozygotes statistically differed between sexes. The annual diversity index of MHC class II DRB-2 alleles significantly decreased along the 16 year study period. Genetic diversity at three microsatellite loci (as neutral markers) was analyzed to separate selective and demographic influences on the MHC class II in this population. Expected heterozygosity was higher than 0.5 in the three microsatellite loci, and all were in Hardy-Weinberg equilibrium.

***Conclusions***

We hypothesize that artificial food provisioning and constrained dispersal due to fencing could result in localized inbreeding and sub-structured populations. These findings support the importance of immunogenetic studies to assess management decisions, especially in isolated ungulate populations.

## Background

The major histocompatibility complex (MHC) is a multigene family of the vertebrate immune system comprised of highly polymorphic loci (Klein 1986). The primary role of the MHC is to recognize foreign proteins, present them to specialist immune cells and initiate an immune response. MHC genes encode cell surface glycoproteins which bind and present antigenic peptides to T cells. The MHC class I (MHCI) genes are expressed on the surface of all nucleated somatic cells and play an essential role in the immune defence against intracellular pathogens by presenting endogenously derived peptides to CD8+ cytotoxic T cells (Klein and Horejsi 1987). MHC class II (MHCII) genes are expressed on antigen-presenting cells of the immune system and present processed exogenous antigens to CD4+ T helper cells.

The MHC contains the most variable functional genes described in vertebrates (Piertney and Oliver 2006). The extensive polymorphism and unusual persistence of alleles at the MHC loci suggests the action of balancing selection, i.e. natural selection works to maintain genetic polymorphism at these loci. Individuals from natural populations deal constantly with a diverse range of pathogens and the polymorphism at MHC loci is what determines the diversity of foreign antigens that the host immune system can recognize to subsequently trigger a specific immune response. Polymorphism at individual loci may result in variable MHCII haplotypes. Recent studies of wild vertebrates suggest further that specific MHCII genotypes confer resistance to a variety of pathogens (Hedrick et al. 2001). In addition to the MHC function in immune response, a large number of studies have reported associations between genetic variation at MHC loci and life history traits (Finch and Rose 1995; Lochmiller 1996; Von Schantz et al. 1996) and behaviour (Penn and Potts 1999) of individuals in natural populations. Therefore, the study of MHC polymorphism has become relevant in ecology and conservation.

Wildlife management for hunting has diverse impacts on the ecology and genetics of vertebrates. It may cause a loss of genetic variation and may lead to short-term reduction of fitness components (Keller and Waller 2002; Altizer et al. 2003). Selective harvests in particular, including trophy hunting, can have important implications for sustainable wildlife management if they target heritable traits. This has been shown in bighorn sheep (*Ovis canadensis*), where declines in mean breeding values for weight and horn size occurred in response to unrestricted trophy hunting (Coltman et al. 2003).

The Iberian red deer (*Cervus elaphus hispanicus*) is a subspecies of red deer inhabiting the Iberian Peninsula. Many red deer populations from South Central Spain have been

managed during the last decades for hunting purposes. Interventions have included fencing, isolation and subsequent disruption of the interconnectivity among populations, along with supplementary feeding and selective hunting. In spite of the current high densities of this game species, such management schemes have probably affected its genetic diversity leading to increased population genetic clustering and to reduced effective population sizes (Slate et al. 2000; Martínez et al. 2002; Zachos et al. 2007). Therefore humans might be selecting unknowingly for rapid changes in the relationships between Iberian red deer and its pathogens (Altizer et al. 2003). In this context, it is important to investigate the genetic diversity of genes that can mediate disease resistance since a reduction in the degree of variation in such genes may have a particularly adverse effect on population viability and conservation.

Here we characterize the polymorphism at the second exon of the b (DRB-2) chain of MHCII in hunter-harvested Iberian red deer from an isolated fenced population in south-central Spain. This red deer population has been managed for hunting purposes and may provide information on the effect of management schemes on the genetic structure and diversity of this species.

## Results

The SSCP and sequence analyses revealed 18 unique DRB-2 sequences among 94 Iberian red deer from a single hunter-managed population in south-central Spain (Fig. 1, Table 1). DRB-2 sequences were confirmed by sequencing the same allele from different individuals and by sequencing several clones from the same PCR. Furthermore, some SSCP conformations were corroborated by F-SSCP analysis of both strands using labeled forward and reverse primers (Fig. 2).

```
           9++ +          +++ +  ++ +  ++        +         + ++         ++  +  ++       +    91
CeelHap1   EYAKSECHFSNGTQRVQFLQRYFYNGEEYVRFDSDWGEFRAVTELGRPDAKYWNSQKEILEQKRAEVDRYCRHNYGVIESFTV
CeelHap2   ***************R**H********F******************V*E*************A**T**************
CeelHap3   **H*R***********G**D********T*********Y***********L**********A**A**T********F*****
CeelHap4   *HL*************Y*D********F**********************************N*******************
CeelHap5   ****************G**D********F*********Y*************************************************
CeelHap6   **H************G**D********F*********Y***********L*********RA**A**T**************
CeelHap7   **Y*R***********G**D***S****T*********Y***********EL**********A*****T********F*****
CeelHap8   **H*G***********G**D*****************Y************F*********RA*****T**************
CeelHap9   *HL************RY*E********F**********************************N*******************
CeelHap10  **H************GY*E**IH****F*********Y**********************R***D**T********F*****
CeelHap11  **H************G**E********F*********Y**********************R******T********F*****
CeelHap12  **P************R**D********F*********Y*********************************************
CeelHap13  **L************GG**E********F**********************************D*****************
CeelHap14  **V************R********R**********************************DN**A**T**************
CeelHap15  ******************H**I**R***A**********************************RA*****TV**********
CeelHap16  ***************R**D********F*********Y*********************************************
CeelHap17  ***************R**D*********************************************A**T**************
CeelHap18  ******************D*********************************************R******T**************
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Figure 1. Amino acid sequence alignment of 18 Iberian red deer DRB-2 alleles.
The standard one-letter amino acid code was used. Numbering of amino acid positions was based on white-tailed deer *OdviDRB* sequences (Van Den Bussche et al. 1999). Asterisks denote amino acids identical to *CeelHap1*. Variable amino acid positions are indicated with a cross (+).

Table 1. Allelic frequencies (2n=188) and haplotype frequencies (n= 94 individuals) for the 18 alleles found.

| Allele | Frequency | Percent | Haplotype | Frequency | Percent |
|---|---|---|---|---|---|
| 5 | 35 | 18.62 | 5-6 | 17 | 18.09 |
| 4 | 28 | 14.36 | 4-4 | 12 | 12.77 |
| 6 | 27 | 14.36 | 6-6 | 12 | 12.77 |
| 2 | 20 | 10.64 | 2-2 | 10 | 10.64 |
| 7 | 15 | 7.98 | 7-7 | 7 | 7.45 |
| 1 | 12 | 6.38 | 1-1 | 6 | 6.38 |
| 3 | 11 | 5.85 | 3-3 | 5 | 5.32 |
| 17 | 10 | 5.32 | 17-17 | 5 | 5.32 |
| 8 | 6 | 3.19 | 8-8 | 3 | 3.19 |
| 12 | 4 | 2.13 | 12-12 | 2 | 2.13 |
| 16 | 4 | 2.13 | 16-16 | 2 | 2.13 |
| 18 | 4 | 2.13 | 18-18 | 2 | 2.13 |
| 11 | 3 | 1.60 | 10-10 | 1 | 1.06 |
| 14 | 3 | 1.60 | 11-11 | 1 | 1.06 |
| 10 | 2 | 1.06 | 13-13 | 1 | 1.06 |
| 13 | 2 | 1.06 | 14-14 | 1 | 1.06 |
| 9 | 1 | 0.53 | 3-6 | 1 | 1.06 |
| 15 | 1 | 0.53 | 4-5 | 1 | 1.06 |
| | | | 4-6 | 1 | 1.06 |
| | | | 4-9 | 1 | 1.06 |
| | | | 4-11 | 1 | 1.06 |
| | | | 6-15 | 1 | 1.06 |
| | | | 7-14 | 1 | 1.06 |
| Total | 188 | | Total | 94 | |

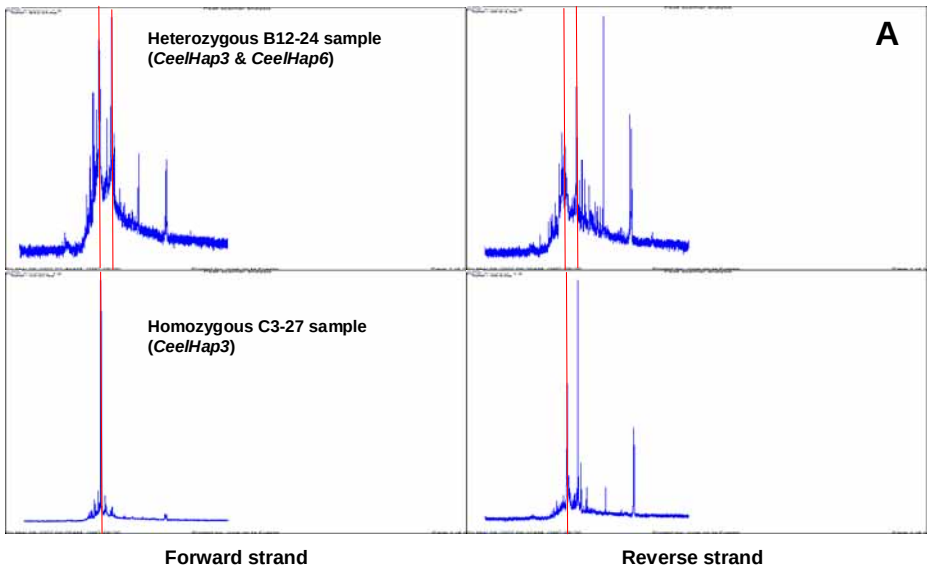


Figure 2. F-SSCP peak profiles of Iberian red deer DRB-2 alleles.

Forward and reverse strands for selected heterozygous and homozygous individuals.

However, although F-SSCP was useful to corroborate homozygous and heterozygous genotypes it was not suitable for distinguishing between different allelic variants.

The analysis of the DRB-2 locus found polymorphic MHCII loci in Iberian red deer. Seventeen percent of nucleotide and 25% of amino acid positions were polymorphic among the 18 alleles identified. Nonsynonymous substitutions occurred at a significantly lower frequency than synonymous substitutions (dN=0.0453±0.0250; dS=0.0761±0.0361; P<0.0001).

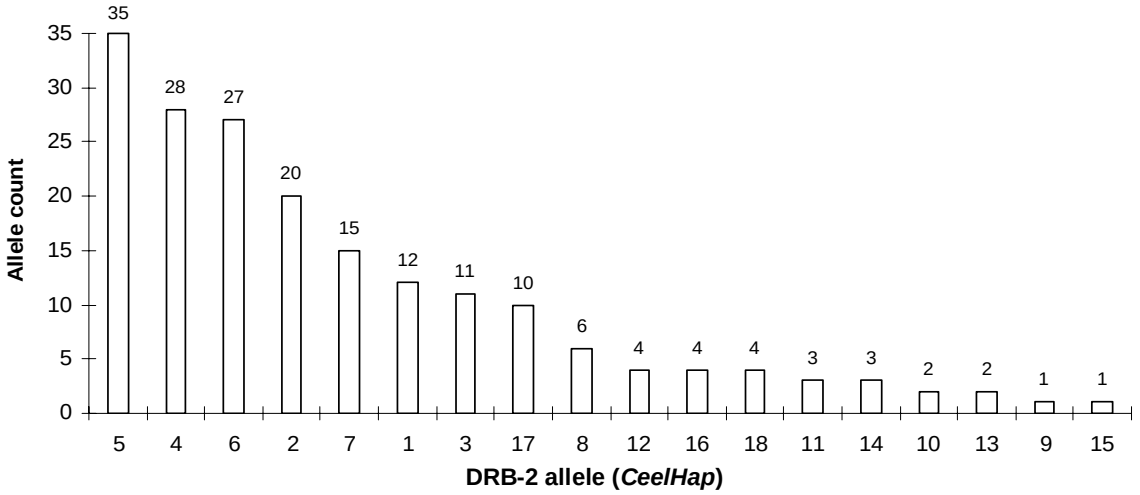


Figure 3. Allelic frequency of Iberian red deer MHCII DRB-2 loci.
Observed frequency of CeelHap alleles (2n = 188).

Allelic frequencies for the 18 unique Iberian red deer alleles ranged from 0.5 % to 18.6 % (Table 1, Fig. 3). Except for alleles *CeelHap*9 and *CeelHap*15, which only appeared once as heterozygotes, the rest of the alleles appeared at least once as homozygotes (Table 1, Fig. 4). Overall, 23 different genotypes out of the possible 170 were found within the 94 sampled individuals, ranging in frequency from 1.1 to 18.1 % (Table 1, Figure 4).

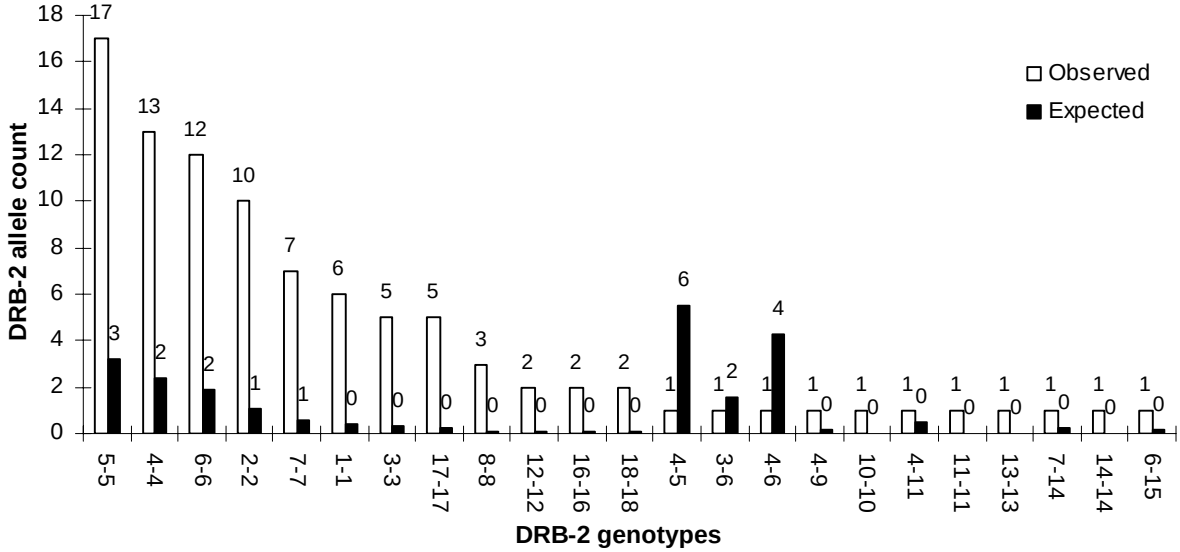


Figure 4. Allelic frequency of Iberian red deer MHCII DRB-2 loci.
Observed versus expected counts of different genotypes for the MHC-II DRB-2 locus in Iberian red deer (n = 94). Only genotypes for which at least one individual was found are shown.

The proportion of genotypes that were found in this deer population differed statistically from expected H-W proportions ($Chi^2$ = 1248.3, d. f. = 153, P<0.001). This disequilibrium was mainly due to homozygous genotypes being observed at much higher proportion than expected (Fig. 4). Observed heterozygosity was 7.5 % (7/94), whereas expected heterozygosity was 89.1 %. Four genotypes out of the 23 genotypes found which appeared as homozygotes represented 55% of the 94 individuals analyzed: *CeelHap2* (*n* = 10, 10.6 %), *CeelHap4* (*n* = 12, 12.8 %), *CeelHap5* (*n* = 17, 18.1 %) and *CeelHap6* (*n* = 12, 12.8 %). The F-SSCP analysis of individuals introduced and hunted before 1991 revealed that only one of the six samples analyzed was heterozygous for the DRB-2 locus (Fig. 5).

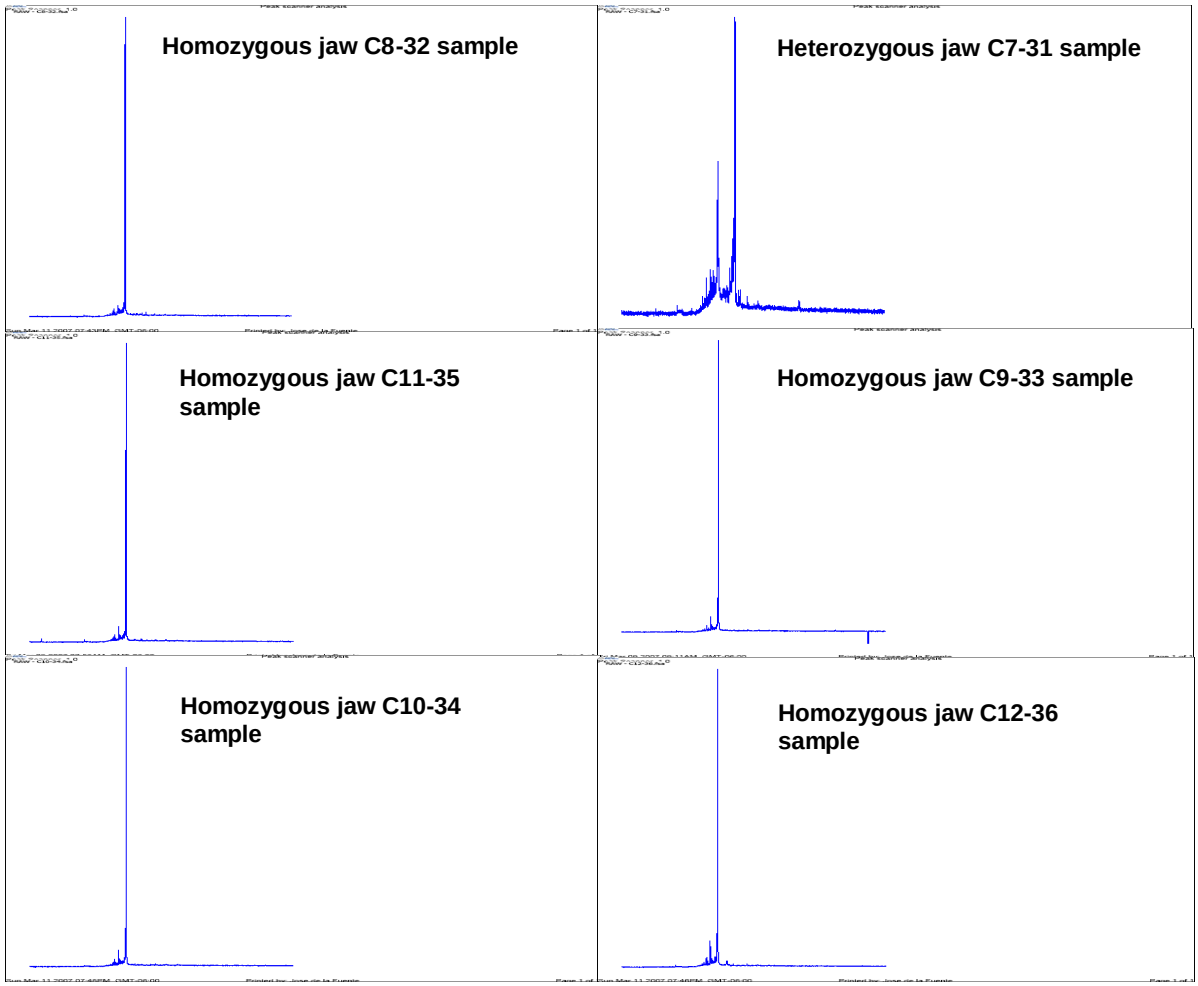


Figure 5. F-SSCP peak profiles of Iberian red deer DRB-2 alleles.
Forward strand for samples derived from individuals introduced and hunted before 1992.

This proportion of heterozygotes did not differ from that found in animals sampled after 1991 ($Chi^2$ = 0.65, d. f. = 1, P = 0.41). All the heterozygotes were males (7 out of 61, 11.5 %), whereas no heterozygotes were evidenced among females.

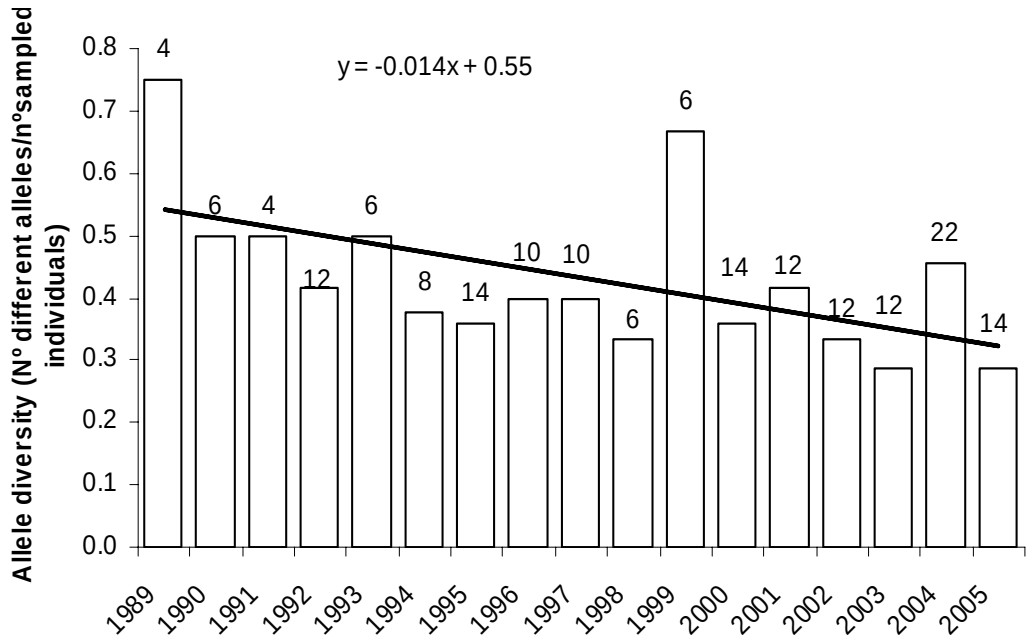


Figure 6. Annual diversity index of MCHII DRB-2 alleles.
Annual diversity for the 18 unique Iberian red deer alleles found according to individual year of birth across the study period.

The proportion of heterozygotes statistically differed between sexes (*Fisher* exact test, P = 0.04). The annual diversity index of MCHII DRB-2 alleles significantly decreased along the study period (2-tailed Spearman correlation, $R_S$ = -0.62, P < 0.01, $n$ = 17 from 1989 onwards; Fig. 6).

Table 2. Allelic frequencies (2n=188) for the alleles found according to individual year of birth across the study period. Year 1988 includes animals born prior to population founding.

| Hapl. | 1988 | 1989 | 1990 | 1991 | 1992 | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 4 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 4 | 2 | 2 | 2 | 4 | 4 |
| 3 | 2 | 0 | 0 | 0 | 2 | 1 | 0 | 4 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 4 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 3 | 0 | 2 | 1 | 4 | 2 | 2 | 0 | 2 | 4 |
| 5 | 2 | 2 | 0 | 0 | 2 | 0 | 2 | 4 | 1 | 0 | 4 | 2 | 0 | 4 | 6 | 0 | 2 | 4 |
| 6 | 0 | 1 | 0 | 0 | 4 | 3 | 0 | 2 | 0 | 4 | 0 | 1 | 2 | 0 | 0 | 6 | 4 | 0 |
| 7 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | 2 | 1 | 2 |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 |
| 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 |
| 12 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 |
| 15 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Nonetheless, no temporal patterns were identified for any single allele, especially when attending to the more frequent ones (Table 2). Phylogenetic analysis of Iberian red deer DRB-2 alleles together with other cervid sequences revealed monophyletic relationships for moose (***AlalDRB***) alleles (Fig. 7). Although some of the Iberian red deer alleles (***CeelHap***) tend to cluster together (***CeelHap4***, 5, 9, 12, 13, 16), ***CeelHap*** alleles were paraphyletic with respect to white-tailed deer (***OdviDRB***) and red deer (***CeelDRB***) sequences (Fig. 7).

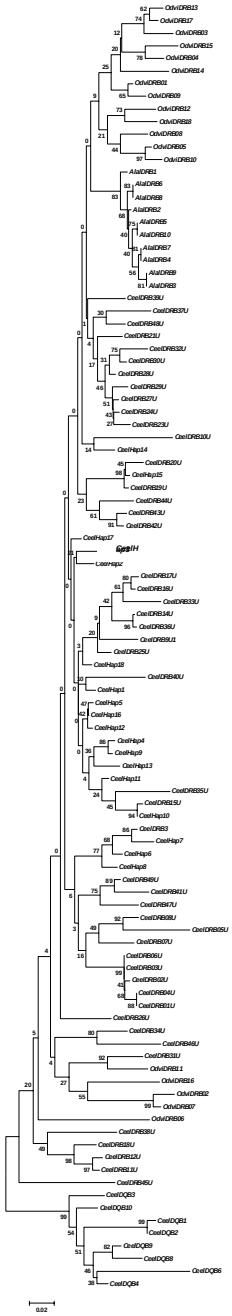

Figure 7. Unrooted neighbor-joining tree of cervid DRB-2 alleles.
DNA sequences of DRB-2 alleles from Iberian red deer (CeelHap; *Cervus elaphus hispanicus*), red deer (CeelDRB; *Cervus elaphus*), white-tailed deer (OdviDRB; *Odocoileus virginianus*), and moose (AlalDRB; *Alces alces*) were included in the analysis.

Genetic diversity at three microsatellite loci was analyzed as genetic reference of neutral markers to separate selective and demographic influences on the MHCII in this population (Table 3). Expected heterozygosity ($H_O$) was higher than 0.5 in all three microsatellite loci (Table 3). Microsatellite loci CSSM22, ETH225 and CSSM19 were in H-W equilibrium at the $\alpha = 0.01$ level with a sequential Bonferroni correction ($P<0.003$).

Table 3. Charactetization of the microsatellite loci used in this study. Genotyping was performed on 96 deer DNA samples as described by Frantz et al. (2006). Abbreviations: A, number of alleles, HE, expected heterozygosity; HO, observed heterozygosity. The test for Hardy-Weinberg equilibrium was performed at each locus at the $\alpha = 0.01$ level with a sequential Bonferroni correction ($P<0.003$).

| Micro. | Dye | Primer sequences 5'-3' | Allele size range (bp) | A | $H_E$ | $H_O$ | H-W equilibrium |
|---|---|---|---|---|---|---|---|
| CSSM22 | 6-Fam | F: TCTCTCTAATGGAGTTGGTTTTTG<br>R: GTTTCTTATATCCCACTGAGGATAAGAATTC | 213-227 | 4 | 0,52 | 0,50 | Yes |
| ETH225 | 6-Fam | F: ACATGACAGCCAGCTGCTACT<br>R: GTTTCTTGATCACCTTGCCACTATTTCCT | 140-173 | 12 | 0,85 | 0,90 | Yes |
| CSSM19 | Hex | F: TTGTCAGCAACTTCTTGTATCTTT<br>R: GTTTCTTTGTTTTAAGCCACCCAATTATTTG | 140-166 | 10 | 0,81 | 0,79 | Yes |

## Discussion

An isolated population of Iberian red deer managed for hunting purposes in south-central Spain was selected to evaluate the effect of hunting management schemes on the genetic diversity of this species. This deer population has been studied by our group over the last 16 years and the population dynamics are well known (Landete-Castillejos et al. 2004; Vicente et al. 2005). The MHCII DRB-2 locus was selected for genetic analysis because it composes part of the functional important peptide binding groove, which has been shown to be the most polymorphic part in MHCII genes (Klein 1986) and its polymorphisms have been related to variation in parasite burdens in deer (Ditchkoff et al. 2005).

The high levels of allelic diversity found within the studied Iberian red deer population were similar to that found in red deer (Swarbrick et al. 1995) and white-tailed deer (Van Den Bussche et al. 1999). In contrast, the DRB-2 genetic diversity reported in moose and reindeer is greatly reduced (Mikko and Andersson 1995; Mikko et al. 1999; Wilson et al. 2003), possibly due to reduced parasite exposure in boreal ecosystems and/or bottlenecks (Mikko and Andersson 1995; Ellegren et al. 1996; Mainguy et al. 2007.

The number and distribution of polymorphisms within the Iberian red deer DRB-2 amino acid sequence was similar to that detected in white-tailed deer and red deer alleles (Swarbrick et al. 1995; Van Den Bussche et al. 1999). This fact denotes a strong selection pressure on certain amino acids within the DRB-2 sequence. However, in contrast to reports in other deer species (Swarbrick et al. 1995; Van Den Bussche et al. 1999), DRB-2 alleles were not under positive Darwinian selection in the Iberian red deer population analyzed herein. Positive Darwinian selection involves a selective pressure favouring change so that only a small number of mutational events are retained in a population, the retention of mutations being much closer to the rate of mutation. In our sample, synonymous substitutions occurred more frequently than non-synonymous substitutions, suggesting that positive selection is not acting on the selected protein sequence.

It has been suggested that the high level of MHC allelic diversity is primarily maintained by pathogen-driven positive Darwinian selection and may be the underlying reason for MHC-based mating preferences, a mechanism that evolved to avoid inbreeding (Van Den Bussche et al. 1999; Potts et al. 1991; Hedrick 1992; Paterson et al. 1998; Carrington et al. 1999; Lohm et al. 2002; Wegner et al. 2003; Harf and Sommer 2005; Schad et al. 2005). However, the DRB-2 genotype composition of the Iberian red deer in this study cannot be explained by positive Darwinian selection, leaving other factors acting as selection pressures on these individuals. Possible factors are discussed below.

As in the Iberian red deer population studied herein, white-tailed deer (Ditchkoff et al. 2005), Canadian moose (Wilson et al. 2003) and African buffalo (Wenink et al. 1998) populations have been reported to comprise DRB genotypes in H-W disequilibrium with homozygous individuals at higher frequency than expected. Selective advantage of heterozygotes over homozygotes would not explain the extreme deficiency of heterozygotes (Penn et al. 2002).

With our observational data we can only guess which management factors may have caused the strong homozygosity of this particular deer population. Founder effects in the form of the introduction of highly homozygous deer is suggested by the 1991 data, only one of six analyzed founders being heterozygous for DRB-2. Given that the recent samples are also highly homozygous; factors that negatively affect genetic pooling (mixing) in this population may still exist.

One possible hypothesis predicts low observed heterozygosity because of pooling of discrete subpopulations with different allele frequencies that do not interbreed as a single randomly mating unit, even if the subpopulations are in Hardy-Weinberg

equilibrium, i.e. a Wahlund effect (Excoffier 2001). This effect has been suggested in populations of white-tailed deer (Kollars et al. 2004; Van Den Bussche et al. 2002) and moose (Wilson et al. 2003). In the case of the Iberian red deer analyzed in the present study, the underlying causes of population subdivision would be not geographic but behavioural barriers to gene flow, which may be followed by some degree of genetic drift in the subpopulations. Those hypothetical behavioural barriers could be explained by factors such as female philopatry and constrained natal dispersal in young males, which can result in clustering of related individuals, inbreeding and structuring of nuclear genotypes across continuous space (Nussey et al. 2005). Some management factors could promote clustering of breeding in our study deer population. It has been shown that the spatial aggregation of resources (such as the fixed feeding places in this estate) induces the aggregation of females (at least during the period of the day they forage), and therefore males defend territories instead of mobile harems of females (Sánchez-Prieto et al. 2004; Carranza et al. 1990). This in turn may lead to an increase of the level of polygyny and a decrease in the effective population size. However, we would expect a higher proportion of heterozygous individuals than we found if dominant stags mate with a large number of females during the rut season regardless of their degree of relatedness, resulting in dissimilar MHC mating rather than positive non-assortative mating. In contrast, our results would be explained if (i) dominant stags predominantly mate with related hinds (i.e. a, positive non-assortative mating hypothesis) or (ii) non dominant stags mate more often than expected and almost exclusively with related females.

We first consider the positive non-assortative mating hypothesis. Any deviation from H-W equilibrium may indicate assortative mating, which may be positive (increases homozygosity, which is our case) or negative (increases heterozygosity). The latter is commonly found in vertebrates and it is accepted that it occurs because inbreeding avoidance and choice of mating partner are interconnected processes. Although some semi-wild ruminant populations seem to mate randomly, despite the presence of pathogen-induced selection on MHC genes (Paterson and Pemberton 1997; Holand et al. 2007), no evidence for positive non-assortative mating has been previously observed. Therefore, although inbreeding avoidance through mating choice seems not to occur in our study population, positive non-assortative mating may be more a consequence than a cause of the observed MHC patterns, and other underlying factors may be responsible.

We now consider the second non exclusive possibility (the non-dispersing spiker hypothesis). Red deer populations are structured by matrilineal clans with adult males

segregating most of the year (Clutton-Brock et al. 1982). Female red deer are philopatric, which means that offspring, mothers, sisters and aunts remain near their dams for life. At finer spatial scales, female philopatry can result in clustering of related individuals and structuring of nuclear genotypes across continuous space (Kurt et al. 1993; Nussey et al. 2005). Male dispersal from matrilineal clans is suggested as a means to reduce the chance of inbreeding; the inclusive fitness of mothers may benefit most from dispersal of their male offspring, leading to a situation in which mothers force their male offspring to leave (Holzenbein and Marchinton 1992). Nevertheless, male dispersal may be constrained in fenced estates and social pressure may be not sufficient, even attenuated (Shaw et al. 2006). This fact may increase the possibility for within kin breeding if other favourable circumstances occur.

Deer stags typically attempt to sneak matings when young, but switch to defending harems when they grow older (Clutton-Brock et al. 1982). Thus, a possible mechanism explaining increased homozygosis is that food clumping may produce an increase in male harassment towards females (Sánchez-Prieto et al. 2004) due to the presence of a large number of competitive males. Our study population is characterized by intense high quality deer management, and there is a high proportion of mature males (6 to 10 years) that congregate at feeding places during the rut season. Feeding stations in our study area are located in open pasture areas. Harassment avoidance may lead females to use feeding stations of dominant males for short time and almost exclusively for foraging, and favour scrublands where subordinate males, possibly related, may have an increased chance to mate with these females, which would explain increased inbreeding. Our findings could reflect a conflict between hierarchy establishment in adult competitive stags (which is complicated by a probable excess of males at feeding places) and gaining of females for mating. Both are probably performed with difficulty in open areas of our study estate because it could be difficult for adult males to establish hierarchy (i. e. displaying agonistic behaviour) in woodlands. In contrast, due to the food scarcity of the Mediterranean summer in natural unmanaged populations in South Spain, females stay in the few meadows remaining in a stag's exclusively defended territory. There being proportionally fewer adult stags than in most high quality management estates, this may prove a good strategy in terms of hind harvest (Carranza and Valencia 1999).

Finally, a simpler explanation is based on the hypothetical effect of selective hunting on deer genetics. Keeping in mind that annually almost one fourth of the animals are removed from the population following trophy-quality criteria (Fierro et al. 2002), it

seems plausible that the cumulative effect of selective hunting has had an adverse effect on genetic variability of this deer, as has been seen in other ungulates (Coltman et al. 2003). If there is any relationship between heterozygosity at MHC-DRB-2 this locus and individual hunter-targeted traits, then the selective culling of stags could lead to increased or reduced heterozygosity depending on the sense of the relationship. For example, if heterozygosity at this locus relates to large antler size, then we would expect an increase in homozygosity, due to the hunted stags being removed. If there is any relationship between homozygosity at this locus and poor condition or disease susceptibility, then the selective culling of animals in poor condition would lead to increased heterozygosity. Other non-hunting explanations could lie in the presence of a disease in the population for which selective pressure is high and a homozygous locus is an advantage.

In the context of overall low homozigosity in the study population, the proportion of heterozygotes was statistically higher in males (7 out of 61, 11.5 %), than in females (0 %, $n = 34$). In fact, all the detected heterozygotes were males. Different non-exclusive explanations, which cannot be tested in this study, could contribute to this finding. Firstly, heterozigosis may prone more advantageous in males than in females, and therefore selecting heterozygous males. Secondly, sex allocation may be influenced by offspring MHC heterozigosity. Thirdly, hunting strategy, which is undergone using different criteria in males (selective or trophy) but not in females, could favour retaining more heterozygotes among males than in females. Higher sample size and correlational studies with fitness parameters in our study population are needed to elucidate this aspect.

The Iberian red deer, red deer and white tailed deer DRB-2 alleles represented paraphyletic groups. As previously discussed by (Van Den Bussche et al. 1999), this result reflects the high sequence identity that exists among artiodactyls DRB-2 alleles.

It has been suggested that red deer contain two expressed DRB loci (Swarbrick et al. 1995). In the study of a different Iberian red deer population using the same experimental procedures described herein, we found two expressed DRB loci in this species (unpublished results). However, this was not observed in our study, as the SSCP and F-SSCP analyses of Iberian red deer samples evidenced the presence of no more than two predominant bands on each individual, a result that was confirmed with the sequence analysis of cloned DRB-2 amplicons. It is likely that these results reflect the presence of identical sequences in both DRB-2 loci and support a high DRB-2 homozygosis in this Iberian red deer population. Alternatively, unless less likely, it is possible that in some

samples we did not amplify all loci to a level to be detectable by SSCP or cloning and sequencing analyses.

Genetic analysis using neutral microsatellite markers suggested that the results of MHCII DRB-2 characterization reflect selective and not demographic influences on this locus in this population. Values of $H_E$ for the microsatellite loci analyzed in this study were higher than those reported by (Frantz et al. 2006) in a continuous red deer population.

We provided data suggesting that non selective forces may contribute to define the MHC population pattern in a red deer population, and further may lead to the removal of MHC allelic variants. We hypothesised that the effects of intensive management on the mating structure may be highly influential, generating a deficit of MHCII DRB-2 heterozygous haplotypes. This deficit is very rarely found in free breeding mammal populations. As a consequence, non-adaptive changes in MHC genetics constitution may predominate rather than natural selection favouring genetic variants as a function of their biological effectiveness. If this is the case, we have identified a negative consequence for Iberian red deer conservation because host genetic diversity plays an important role in buffering populations against pathogens and widespread epidemics (e. g. Spielman et al. 2004).

A previous study on neutral loci in Iberian red deer suggests that there are differences between red deer populations in South Central Spain due to differing allele frequencies and/or the number of alleles (Martínez et al. 2002), which indicates that genetic goals in the management of Iberian deer populations have to avoid inbreeding to maintain potentially adaptive genetic variation. Previous research confirms that increase of inbreeding in artificially restored red deer populations may easily be higher than the threshold at which natural selection is believed to counteract the fixation of deleterious alleles in the gene pool (Zachos et al. 2007). Although our study population originated from a relatively high number of founders, genetic clustering may increase the probability of allele losses (Figures 3, 4, 6, Table 2). Additional research is needed in many other genes involved in shaping the immune repertoire, either solely or in conjunction with MHC (Acevedo-Whitehouse et al. 2006).

## Conclusions

In conclusion, our findings raise concern about the ecological consequences of the management of wildlife on deer life history. The efficacy of selection in maintaining genetic

diversity can vary substantially with the degree of population subdivision (Muirhead 2001). In particular, management of game species may involve a risk of alteration of their genetic properties (Carranza et al. 2003). Intensive game management situations may be analogous to bottlenecks, which increase the risk of loss of rare alleles and probably the risk of outbreaks of severe diseases as a consequence of decreased MHC variation (Carranza et al. 2003; O'Brien and Evermann 1988). Therefore, although red deer is an abundant and widespread species in Spain, its MHC polymorphism (and overall genetic variability), may be threatened by (i) increasing habitat fragmentation and barriers to gene flow (Carranza et al. 2003), (ii) by any alteration of mating substructures within managed populations favouring positive assortative mating, or (iii) by selective hunting.

## Methods

### Study site and Iberian red deer population

The study was conducted in a 900 ha hunting estate in the province of Ciudad Real, south-central Spain (38º55'N; 0º36´E; 600-850 m above sea level). The habitat is composed of Mediterranean scrublands (mainly evergreen oak *Quercus ilex*) with scattered pastures and small crops (Vicente et al. 2004; Acevedo et al. 2007). The climate is Mediterranean and most annual rainfall is concentrated between September and May. The dry season is characterized by high temperatures. Deer numbers were estimated yearly through repeated direct counts at the feeding places at the end of July and during the rutting season and resulted in a mean±SD of 306±27 individuals with a mean density of 0.35 individuals/ha. The sex-ratio was 1 to 1.3 hinds per male in September and the population density moderately increased during the study period (2000-2006) from 0.30 ind/ha in 2000 to 0.43 ind/ha in 2006.

Deer reproduce naturally in the estate and should be regarded as one single population where no introduction of individuals has been performed since 1991. In 1989, the estate had 5 females and 2 calves. Between 1989 and 1991, 126 individuals (85 females and 41 males) were introduced from three different locations. Management schemes for hunting purposes in the estate include fencing in order to restrict movement of wildlife, artificial water holes, supplemental feeding in troughs, and selective hunting of a mean of 80 ± 13 deer each year (8.89 per square km), mostly by the same single hunter. Criteria for selective shooting of deer included obvious poor condition in both sexes, lack of a fawn in females, and poor trophy characteristics in males (e.g. short spikes in yearlings or lack of

second tines in stags, Fierro et al. 2002). Prime males were shot as trophies only after participating in several rutting seasons.

**Sample collection and DNA extraction**

Samples were collected from 94 selectively harvested Iberian red deer (34 females and 60 males) between 2000 and 2006, during the main hunting season from October to February. This sample represented approximately 30% of the population living in the estate. Lymph node tissue fragments of approximately 2 $cm^3$ were prepared and stored at -80ºC for DNA extraction and genetic analyses. Genomic DNA was extracted from mesenteric lymph node samples using Tri Reagent (Sigma, St. Louis, MO, USA) and following manufacturer's recommendations.

DNA was extracted from lower jaw bone remains of 6 deer introduced and hunted before 1992. DNA was extracted from 2 $cm^2$ jaw sections following the procedure described by (Lleonart et al. 2000) for human remains.

**Single-stranded conformation polymorphism (SSCP) analyses**

The DRB-2 locus was amplified by polymerase chain reaction (PCR) using primers LA31 and LA32 (Sigurdardottir et al. 1991) in a 50-µl volume PCR (1.5 mM $MgSO_4$, 0.2 mM dNTP, 1X AMV/*Tfl* reaction buffer, 5u *Tfl* DNA polymerase) employing the Access RT-PCR system (Promega, Madison, WI, USA). Reactions were performed in an automated DNA thermal cycler (Techne, Cambridge, England, UK) PCR machine (model TC-512) for 35 cycles. After an initial denaturation step of 2 min at 95ºC, each cycle consisted of a denaturing step of 1 min at 95ºC, an annealing for 30 sec at 50ºC and an extension step of 1 min at 68ºC. Negative control reactions were performed with the same procedures, but adding water instead of DNA to monitor contamination of the PCR. The allelic diversity of DRB-2 was characterized using a modification of the single-stranded conformation polymorphism (SSCP) analysis (Van Den Bussche et al. 1999), without isotopic labelling of PCR products and using gel silver staining (Amersham Biosciences, Sweden).

A sub-sample of the heterozygous and homozygous individuals identified by SSCP and the deer introduced and hunted before 1992 were also analyzed by fluorescent SSCP (F-SCCP). Samples were prepared for F-SSCP following the procedure of Lento et al. (2003) using FAM-labeled primers and the ABI 310 manual (Applied Biosystems). A 2µl aliquot of a 1:5 dilution of the *DQB* amplicon in ddH2O was mixed with 10.5 µl deionized formamide (Gibco), 0.5 µl of freshly made 0.3 N NaOH and 1.0 µl GeneScan-500 TAMRA dye standard (ABI Prism). The samples were denatured at 96°C for 2–4 min and then

snap-cooled on wet ice slurry for 2–4 min before electrophoresis on an ABI 310 automated capillary sequencer running 3% GeneScan (ABI Prism) polymer in 1×TBE. Runs were conducted for 18 min and the heating mantle set at a constant temperature of 29°C. SSCP peak profiles were analyzed using ABI 310 data collection and peak scanner software (v1.0). The relative position of each SSCP peak was calculated with peak scanner by standard interpolation between peaks of the TAMRA standard run in each lane. As in manual SSCP, this relative position does not represent an absolute size of the fragment and was used only to identify unique alleles across runs. The F-SSCP profile was analyzed on both strands by comparing the results obtained with forward- and reverse-labelled primers.

**Sequence analyses**

All unique SSCP conformations were cloned for subsequent sequence analysis. Amplified fragments were resin purified (Wizard, Promega) and cloned into the pGEM-T vector (Promega) for sequencing both strands by double-stranded dye-termination cycle sequencing (Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University). At least five independent clones were sequenced for each cloned allele.

For phylogenetic analysis of the 18 Iberian red deer DRB-2 alleles (*CeelHap*) identified here, we included published DNA sequences of 56 DRB-2 alleles from red deer (*CeelDRB*; Swarbrick et al. 1995), 18 DRB-2 alleles from white-tailed deer (*OdviDRB*; Van Den Bussche et al. 1999), and 10 DRB-2 alleles from moose (*AlalDRB*; Mikko and Anderson 1995). Multiple sequence alignment was performed using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA) with an engine based on the Clustal W algorithm (Thompson et al. 1994). Nucleotides were coded as unordered, discrete characters with five possible character-states: A, C, G, T, or N and gaps were coded as missing data. Phylogenetic analyses were implemented using MEGA version 3.0 (Kumar et al. 2004). Genetic distances among all DRB-2 alleles were calculated based on Kimura two-parameter model. A neighbor-joining phylogenetic tree (Saitou and Nei 1987) was constructed under the minimum evolution criteria with equal weights for all characters and substitutions, heuristic searches with 5 random additions of input taxa and tree bisection-reconnection (TBR) branch-swapping. Stability or accuracy of inferred topology(ies) were assessed via bootstrap analysis (Felsenstein 1985) of 1000 replications. Relative frequencies of nonsynonymous (dN) and synonymous substitutions (dS) within and among all pairwise comparisons of Iberian red deer DRB-2 alleles were estimated

following the method of Nei and Gojobori (Nei and Gojobori 1986) and applying the Jukes and Cantor (Jukes and Cantor 1969) correction for multiple substitutions.

The detection of 18 alleles among the 94 individuals sampled in this population indicated 170 possible DRB-2 genotypes. A $Chi^2$ test was performed to evaluate whether the observed heterozygosity was in H–W equilibrium, using GenAlEx 6 Software (Peakall and Smouse 2006). As an estimation of MHC DRB-2 loci diversity, we used the number of different alleles identified in relation to the number of individuals studied. The level of significance was established at P=0.05.

**Genotyping with microsatellite loci**

Genotyping was performed on 96 deer DNA samples extracted as described above using microsatellite loci CSSM22, ETH225 and CSSM19 (Frantz et al. 2006). To avoid linkage, microsatellite loci from different chromosomes were chosen in this study (http://www.marc.usda.gov/genome/cattle/references/twintable.html). None of these loci is linked to MHC. In order to avoid noise from cariable adenylation during the PCR, the "pigtail" sequence GTTTCTT was added to the 5'-end of each reverse primer (Brownstein et al. 1996). The multiplex PCR was done with labelled forward (F) oligonucleotide primers (Table 3) in a 50-µl volume (1.0 mM $MgSO_4$, 1 X avian myeloblastosis virus (AMV) RT/*Thermus flavus* (*Tfl*) reaction buffer, 0.2 mM each deoxynucleoside triphosphate (dNTP), 5 u *Tfl* DNA polymerase, 0.22, 0.09 and 0.12 µM of each oligonucleotide primer for microsatellite CSSM22, ETH225 and CSSM19, respectively) employing the Access RT-PCR system (Promega, Madison, WI, USA). Reactions were performed in an automated DNA thermal cycler (Techne model TC-512, Cambridge, England, UK). The PCR consisted of an initial step of 5 min at 95ºC followed by 37 cycles of a denaturing step of 45 sec at 95ºC and annealing step of 60 sec at 53 ºC and an extension step of 45 sec at 68 ºC. The reaction was terminated after a final extension at 68ºC for 10 min. Control reactions were done using the same procedures, but without DNA added to control contamination of the PCR reaction. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (1 Kb Plus DNA Ladder, Promega, Madison, WI, USA). Fragments were separated using an ABI 3730 authomated DNA sequencer (Applied Biosystems, Inc. Foster City, CA, USA) and sized relative to a ROX-labelled size marker with bands of known size (G500LIZ -250). The data were analyzed using program Peak Scanner (Applied Biosystems).

The expected ($H_E$) and observed ($H_O$) heterozygosity were estimated with GENETIX 4.05.2 (Belkhir et al. 1996-2004). The exact test for deviation from Hardy-Weinberg (H-W) equilibrium at each locus was performed with GENEPOP on the Web v1.2 (http://genepop.curtin.edu.au/genepop_op1.html; Raymond and Rousset 1995) and significance was tested with 10000 dememorizations, 1000 batches, and 10000 iterations.

## Author's contributions

IGFM and JV generated the molecular data and performed the data analyses. IGFM, JMPL and VN carried out the laboratory work. AJM participated to data analysis. YF provided all the animals of the study and helped with the first processing of them. IGFM, JV, JF and CG wrote the manuscript. JF and CG conceived the study and participated in its desing and coordination. All authors helped to draft the manuscript, also all of them read and approved the final manuscript.

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## Relación del complejo mayor de histocompatibilidad de la clase II con infecciones y algunos indicadores de condición en el ciervo ibérico


### Resumen

La diversidad genética del hospedador juega un importante papel en poblaciones naturales, tamponando la acción de los patógenos. En este trabajo se caracteriza la diversidad alélica en el segundo exón de la cadena DRB-2 del complejo mayor de histocompatibilidad (MHC) clase II en una población de ciervo ibérico (*Cervus elaphus hispanicus*) en relación a infecciones de naturaleza diversa (macroparásitos y un microparásito causante de tuberculosis) y de algunos indicadores de condición relevantes de la historia natural de la población (tamaño del bazo y engrasamiento). También se estudia la hipótesis de que la calidad del MHC debería reflejarse en características dependientes de la condición.

Ningún haplotipo confirió una resistencia general o una mayor susceptibilidad contra la totalidad de infecciones. Se observaron correlaciones específicas significativas entre algunos de los alelos del DRB-2 y determinadas infecciones. Se detectaron relaciones entre los alelos del DRB-2, la condición física y el tamaño del bazo.

Estos resultados demuestran un significado funcional de los genes del MHCII en la protección del ciervo ibérico contra los patógenos, revelando que la variación en un sólo locus del MHCII DRB es la base de la diferente resistencia contra la gama de patógenos encontrados en este complejo escenario epidemiológico. Nuestros hallazgos correlacionales también apoyan la idea del papel del MHCII como un elemento genético en la mejora de la condición, lo que se evidencia por los efectos de los parásitos sobre los indicadores fisiológicos con una base genética.

Se concluye que los estudios inmunogenéticos del MHC son de importancia a la hora de tomar decisiones sobre las medidas de manejo en poblaciones de ciervo, ya que (i) la pérdida genética puede conducir al aumento en la aparición de diversas enfermedades, y (ii) las infecciones y la condición física pueden reflejar el estado genético de las poblaciones. Habría que discutir así las medidas de manejo diseñadas para mantener la variación del MHC, lo que proporcionaría una ventaja genética contra las diversas enfermedades en el ciervo ibérico.

**Major histocompatibility complex class II polymorphism in relation to infections and life history traits of management relevance in Iberian red deer**

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## Abstract

1. Host genetic diversity plays an important role in buffering populations against pathogens. We characterized the allelic diversity at the second exon of the b (DRB-2) chain of the major histocompatibility complex class II (MHCII) locus in a population of Iberian red deer (*Cervus elaphus hispanicus*) in relation to infections of diverse nature (macroparasites and a microparasite causing tuberculosis) and relevant life history traits (spleen size and body condition). We also explored the hypothesis that MHC quality should be apparent in expression of condition-dependent traits.
2. No haplotype conferred general resistance or susceptibility against the overall plethora of infections. There were specific significant correlations between some DRB-2 alleles and specific infections. We detected associations between DRB-2 alleles and body condition and spleen size.
3. Our results evidenced a functional significance of MHCII genes in the defence of Iberian red deer against pathogens, revealing that variation in a single MHCII DRB locus differently underlies resistance against the range of pathogens found in such complex epidemiological scenarios. Our correlational findings also support a role of MHCII as a fitness-enhancing genetic element which is mediated by parasite effects on life traits with genetic basis.
4. We conclude that MHC immunogenetic studies are relevant to assess management decisions in red deer populations since (i) genetic loss may lead to increased disease occurrence, and (ii) infection and life history traits may reflect genetic status of populations. We discuss in the context of management schemes designed to maintain MHC variants that provide a genetically advantage against diseases in the Iberian red deer.

**Key words**: Wildlife disease, life history trait, major histocompatibility complex, parasite, red deer, tuberculosis.

## Introduction

The major histocompatibility complex (MHC) is a multigene family comprised by highly polymorphic loci of the vertebrate immune system (Klein 1986). The primary role of the MHC is to recognise foreign proteins, present them to specialist immune cells and initiate an immune response. MHC genes encode cell surface glycoproteins which hind and present antigenic peptides to T cells. The MHC class I (MHCI) genes are expressed on the surface of all nucleated somatic cells and play an essential role in the immune defence against intracellular pathogens (such as viruses) by presenting endogenously derived peptides to CD8+ cytotoxic T-cells (Klein 1996). MHC class II (MHCII) genes are expressed on antigen-presenting cells of the immune system and present processed exogenous antigens to CD4+ T-helper cells. Class II molecules are associated with immune response derived from extracellular pathogens (most bacteria, nematodes, cestodes; Villadangos 2001).

The MHC contains the most variable functional genes described in vertebrates (Piertney and Oliver 2006). The extensive polymorphism and unusual persistence of alleles at the MHC suggests the action of balancing selection, which means that somehow natural selection works to maintain genetic polymorphisms (for a review of the main types of balancing selection in humans and other vertebrates see Takahata and Nei 1990; Hedrick 1999; Penn et al. 2002). It have been suggested that the high level of MHC variability is primarily maintained by parasite (pathogen)-driven selection (e. g. Paterson et al. 1998; Carrington et al. 1999; Lohm et al. 2002; Wegner et al. 2003; Harf and Sommer 2005; Schad et al. 2005). In contrast, directional selection favours a single allele and non selective forces, such as genetic drift and inbreeding depression, would reduce genetic variation in MHC and increase susceptibility to diseases (Sommer 2005; The authors, submitted).

Individuals from wild populations deal constantly with a diverse range of pathogens and the polymorphism at MHC loci is what determines the diversity of foreign antigens that the host immune system can recognize and subsequently trigger a specific immune response. Polymorphism at individual loci may result in variable MHCII haplotype composition. Recent studies suggest further that specific MHCII genotypes may confer resistance to a variety of pathogens in wild vertebrates (Hedrick et al. 2001). In particular, some MHCII haplotypes have been associated with macroparasite rates in ruminants (Paterson et al. 1998; Ditchkoff et al. 2005), which indicates the existence of functional significance of MHCII genes in defence against macroparasites. Although *Mycobacterium tuberculosis* complex are intracellular pathogens, CD4+ T cells are critical for control of

micobacterial infections in animals and humans and they are activated when mycobacterial antigens are processed and presented by MHCII molecules (Noss et al. 2000; Amirzargar et al. 2004; Torres et al. 2006). Most of these studies found associations between certain MHC alleles and infection with single viral, bacterial or parasitic agents (e. g. Paterson et al. 1998; Meyer and Thomson 2001; Wegner et al. 2003), but little is known for multipathogen-one host systems, especially when pathogens of diverse nature are involved (Meyer-Lucht and Sommer 2005), which is the common situation in wild population naturally exposed to a wide range of pathogens. Studies combining two or more pathogens would be very valuable to improve the understanding of MCH variability in wildlife species, and would provide support for management decisions depending on the life history or conservation importance of a given host-pathogen system.

In addition to the MHC function in immune response, a large number of studies have reported associations between genetic variation at MHC loci and life history traits (e. g. Finch and Rose 1995; Lochmiller 1996) and behaviour (kin recognition, inbreeding avoidance, mate choice; e. g. Penn and Potts 1999) of individuals in natural populations. This confers an extra-value to the study of MHC and pathogens in applied ecology and conservation. Therefore, empirical studies examining MHC type and parasite load in concomitance with life history traits of ecological relevance are needed.

Wildlife management for hunting often causes a loss of genetic variation of vertebrates and may lead to short-term reduction of fitness components (Keller and Waller 2002; Altizer et al. 2003) and disease susceptibility (Acevedo-Whitehouse et al. 2005). The Iberian red deer (*Cervus elaphus hispanicus*) is a subspecies of red deer inhabiting the Iberian Peninsula. Many red deer populations from South Central Spain have been managed during the last decades for hunting purposes, which included fencing, isolation and subsequent disruption of the interconnectivity among populations. In spite of the current high densities of this game species, such management schemes have probably affected the genetic diversity of the red deer, and have lead to increased population genetic clustering and to reduced effective population sizes (Slate et al. 2000, Martínez et al. 2002, Zachos et al. 2007). Since humans might be selecting unknowingly for rapid changes in the relationships between red deer and its pathogens (Altizer et al. 2003) and the reduction in the degree of variation in such genes may have a particularly adverse effect on population viability and conservation, it is imperative investigating the diversity of genes that can mediate disease resistance in the Iberian red deer.

We compared MHCII DRB-2 locus polymorphism with data on infections with different pathogens and life history traits obtained from hunter-harvested Iberian red deer. The objectives of this research were (i) to study the relationships between DRB-2 polymorphism and pathogen infection, (ii) to assess the relationships between DRB-2 polymorphism and life history traits such as body condition and spleen size, and (iii) to explore the hypothesis that MHC quality should be apparent in expression of condition-dependent traits.

## Materials and Methods

*Study site and animals*

The study was conducted in a 900 ha hunting estate in the province of Ciudad Real, south-central Spain (38º55'N; 0º36´E; 600-850 m above sea level). The habitat is composed of Mediterranean scrublands (mainly evergreen oak Quercus ilex) with scattered pastures and small crops (Vicente et al. 2004; Acevedo et al. 2007). The climate is Mediterranean and most annual rainfall is concentrated between September and May. The dry season is characterized by high temperatures. Deer numbers were estimated yearly through repeated direct counts at the feeding places at the end of July and during the rutting season and resulted in a mean±SD of 306±27 individuals with a mean density of 0.35 individuals/ha. The sex-ratio was 1 to 1.3 hinds per male in September and the population density moderately increased during the study period (2000-2006) from 0.30 ind/ha in 2000 to 0.41 ind/ha in 2005.

Data were collected between 2000 and 2005 from 94 hunter harvested red deer. Sex and total body length to the nearest 0.1 cm and were recorded. The spleens were collected and the mass was measured to the nearest 0.1 g using an electronic-digital scale (Vicente et al. 2007a, b). Body condition was estimated using the kidney fat index (KFI) since whole body fat levels in cervids highly correlate with KFI (Finger et al. 1981). KFI is defined as the percent of fat weight that surrounds the kidney in relation to kidney weight. The age of deer was determined by histology from incisors (Matson´s Laboratory, Montana). For statistical purposes, animals where grouped in age classes as follows: (1) calves (<1 year), (2) yearlings (1-3 years), (3) subadults (3-4 years) and (4) adults (≥ 4 years).

*Parasitism quantification and TB diagnosis*

The multi host-parasite system analyzed is endemically persistent in red deer populations from South Central Spain (e.g. Vicente et al. 2006; 2007a). Namely, the abundance of 2 different Metazoan parasite taxa and the presence of one bacterial disease were determined. *Elaphostrongylus cervi* (Nematoda: Protostrongylidae) is widespread in Spanish populations of red deer (Vicente and Gortázar 2001; Vicente et al. 2006), and adults are found in the fascia and connective tissue around skeletal muscles. Fresh faecal samples were collected directly from the rectum during field necropsy and first-stage (L1) larvae were extracted in less than 24 h from 8 g of faeces (Forrester and Lankester 1997) and were expressed as number of larvae per gram of faeces. The head, neck, ears and ventral surface of animals were inspected for tick counting and collection. Tick species (Acari: Ixodidae) in the study area included *Hyalomma m. marginatum*, *Rhipicephalus bursa, Hy. lusitanicum* and *D ermacentor marginatus* (de la Fuente et al, 2004a; Ruiz-Fons et al. 2006). Parasite abundance was defined as mean number of ticks (larvae, nymphs and adults) per deer.

*Mycobacterium bovis* infection was diagnosed by the presence of granulomatous lesions, which was confirmed by culture and spoligotyping of mycobacteria (Gortázar et al. 2005; Vicente et al. 2006). For this purpose, parotidean, retropharyngeal and submandibular lymph nodes in the head, tracheobronchial and mediastinic lymph nodes and lungs in the thorax, and hepatic and mesenteric lymphnodes, ileocecal valve, kidneys, liver and spleen in the abdomen were analyzed for granulomatous lesions. Pools of lymph nodes samples were cultured in Coletsos medium (Bio-Mérieux, Marcyl'Étoile, France) as described previously (Gortázar et al. 2005). Animals with lesions and positive cultures were classified as positive for tuberculosis (TB) infection.

*Genetics*

The sample ($n$=94) represented approximately 30% of the population living in the estate. Lymph node tissue fragments of approximately 2 cm3 were prepared and stored at -80ºC for DNA extraction and genetic analyses. Genomic DNA was extracted from mesenteric lymph node samples using Tri Reagent (Sigma, St. Louis, MO, USA) and following manufacturer's recommendations. The DRB-2 locus was amplified by polymerase chain reaction (PCR) using primers LA31 and LA32 (Sigurdardottir et al. 1991) in a 50-µl volume PCR (1.5 mM $MgSO_4$, 0.2 mM dNTP, 1X AMV/Tfl reaction buffer, 5u Tfl DNA polymerase) employing the Access RT-PCR system (Promega, Madison, WI, USA). Reactions were performed in an automated DNA thermal cycler (Techne, Cambridge,

England, UK) PCR machine (model TC-512) for 35 cycles. After an initial denaturation step of 2 min at 95ºC, each cycle consisted of a denaturing step of 1 min at 95ºC, an annealing for 30 sec at 50ºC and an extension step of 1 min at 68ºC. Negative control reactions were performed with the same procedures, but adding water instead of DNA to monitor contamination of the PCR. The allelic diversity of DRB-2 was characterized using a modification of the single-stranded conformation polymorphism (SSCP) analysis (Van Den Bussche et al. 1999), without isotopic labelling of PCR products and using gel silver staining (Amersham Biosciences, Sweden).

*Statistical analyses*

Eighteen alleles were detected among 94 individuals sampled from this population. To guarantee statistical power, analyses were performed for the most frequent ($n \geq 8$) DRB-2 alleles. In our statistical models, individual allele was fitted as categorical explanatory variable with four levels and we tested for associations with (i) TB infection status (as categorical binary response variable, 0 = negative, 1 = positive), (ii) parasite abundances (faecal *E. cervi* L1 counts and ticks; respectively), and (iii) life-traits (body condition as KFI and spleen mass, as continuous explanatory variables, respectively). We also included as categorical explanatory variables sex, age (excluding calves), season (defined as wet season from October to March, and dry season for the rest of the year), and the interactions of alleles by season since differences between genetic groups could arise only during a particular season (Vicente et al. 2005; Ruiz-Fons et al. 2006). In the case of the spleen model, we added the continuous explanatory variable body length to control for body size effects. Sampling year (2000-2005) was incorporated as random categorical effect.

We tested the relationships between infections and life traits; and between life traits (body condition and spleen mass). We firstly tested the relationships KFI (as response variable) and each parasite abundance (as explanatory continuous variables), respectively, and TB presence (as explanatory categorical variable). Secondly, we tested the relationships between each pathogen (parasite abundances and TB presence as response variables) and spleen mass (as explanatory categorical variable). Finally, we tested the relationship between spleen (continuous response variable) and KFI (continuous explanatory variable). All these models controlled for sex, age and body length; and year was included a random factor.

The models for which the binary response variable was TB status were build with a binomial distribution and analysis carried out on the logit transform, whereas a Poisson distribution and a log link was used for the remaining analyses (parasite abundances and life

traits, Wilson and Grenfell 1997). We used a Poisson distribution and a log link because (i) the spread in the residuals increased for the larger fitted values and (variance increases with the mean) and (ii) the absence of normality in dependent variables (Wilk-Shapiro and Lilliefors tests). We controlled for overdispersion when the overdispersion parameter of the model (deviance/d.f.) was > 1. All analyses were carried out as generalised linear mixed models (GLMMs) in SAS (Glimmix Procedure; SAS version 9.1.3. SAS Institute Inc., Cary, NC, USA). Statistics concerning infections and life traits are shown as least squares means of the respective models, and therefore the partial effects of the other explanatory variables upon the selected ones are accounted for. Paired *T*-test comparisons between the least squares means of the different haplotypes were performed. We performed $Chi^2$ to tests whether the observed heterozygosity did agree with the Hardy–Weinberg equilibrium using GenAlEx 6 Software (Peakall and Smouse 2006). The level of significance was established at 5 %.

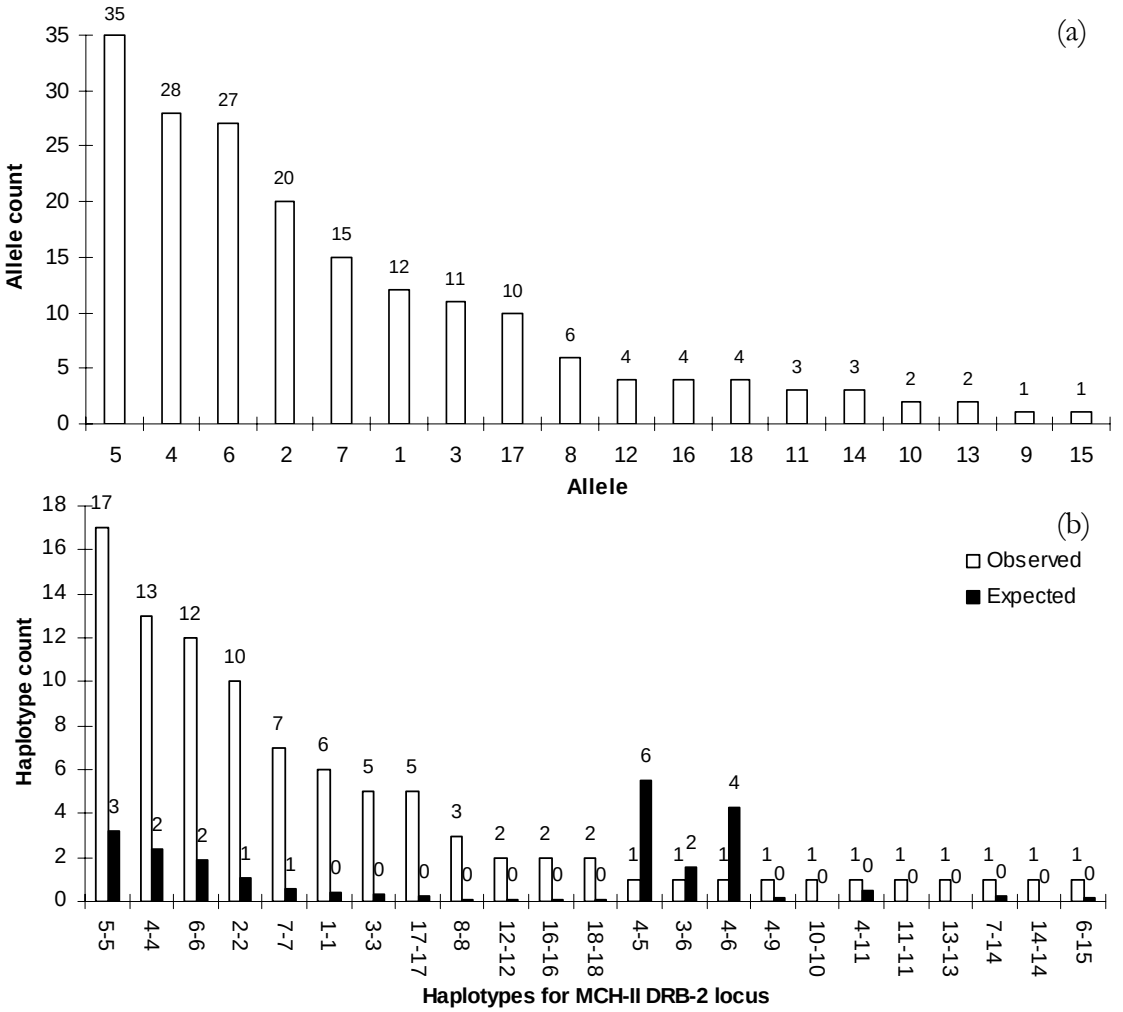


Figure 1. (a) Observed frequency of alleles ($2n$ = 188). (b) Observed *vs* expected counts of different haplotypes for MHC-II DRB-2 locus in red deer ($n$ = 94). Absent haplotypes (for which also there exist expected values) are not graphically shown since a total of 170 different combinations are possible. Observed counts are indicated at the top of the bar.

## Results

*Descriptive*

The SSCP and sequence analyses revealed 18 unique DRB-2 sequences among 94 Iberian red deer from a single hunter-managed population in south-central Spain (Fig. 1). DRB-2 sequences were confirmed by sequencing the same allele from different individuals and by sequencing several clones from the same PCR. The proportion of genotypes that were found in this deer population differed statistically from expected Hardy–Weinberg proportions ($Chi^2$ = 1248.3, d. f. = 153, $p$<0.001). This disequilibrium was mainly due to homozygous genotypes being observed at much higher proportion than expected (Fig. 1). Observed heterozygosity was 7.5 % (7/94), whereas expected heterozygosity was 89.1 %. Four genotypes out of the 23 genotypes found which appeared as homozygotes represented 55% of the 94 individuals analyzed: *CeelHap2* ($n$ = 10, 10.6 %), *CeelHap4* ($n$ = 12, 12.8 %), *CeelHap5* ($n$ = 17, 18.1 %) and *CeelHap6* ($n$ = 12, 12.8 %).

**Table 1**. Prevalence of tuberculosis and parasitic infections, and mean abundance of such parasites of red deer (excluding calves) from a population in Southwestern Spain with different MCH haplotype. Sample size is indicated.

| | **Prevalence ± S.E. (*n*)** | | | | **Mean abundance ± S.E** | | | |
|---|---|---|---|---|---|---|---|---|
| | ***CeelHap2*** | ***CeelHap4*** | ***CeelHap5*** | ***CeelHap6*** | ***CeelHap2*** | ***CeelHap4*** | ***CeelHap5*** | ***CeelHap6*** |
| **Tuberculosis** | 10.0±10.0 (10) | 16.7±11.0 (12) | 5.88±6.0 (17) | 25.0±13.0 (12) | --- | --- | --- | --- |
| **Ticks** | 30.0±15.3 (10) | 58.3±14.9 (12) | 58.8±12.3 (17) | 66.7±14.2 (12) | 1.0±0.6 | 21.8±9.4 | 10.7±4.5 | 13.6± 4.7 |
| ***E. cervi*(L1)** | 20.0±13.3 (10) | 58.3±14.9 (12) | 46.7±13.3 (15) | 41.7±14.9 (12) | 1.2±1.0 | 19.8±11.6 | 7.7±3.0 | 3.5±1.6 |

The association between DRB-2 genotypes and infections were estimated including the 4 most common alleles (Table 1, Fig. 1). Whereas ticks and counts of *E. cervi* L1 were widely spread across the individuals, TB infection was more restricted, only reaching

comparable figures to those of parasites in individual with *CeelHap5*. Average values for spleen size and body condition in relation to the haplotype are shown in Table 2.

**Table 2.** Mean spleen mass (g) and body condition (kidney fat index, as %) of red deer (excluding calves) from a population in Southwestern Spain with different MCH haplotype. Standard error and sample size (*n*) are indicated. It should be noted that values do not account for the partial effects of the other explanatory variables sex, age, body size and season (Table 4, Figure 2b).

| | ***CeelHap2*** | ***CeelHap4*** | ***CeelHap5*** | ***CeelHap6*** |
|---|---|---|---|---|
| **Spleen mass (g)** | 320.0±32.0 (10) | 338.6±34.8 (12) | 343.5±31.4 (15) | 416.7±35.3 (11) |
| **KFI (%)** | 51.1±13.0 (8) | 44.9±10.7(11) | 46.2±7.8 (17) | 46.0±7.0 (12) |

*Associations between MHC-II genotypes and infections*

After controlling by epidemiological predictors and body size, TB infection, the abundance of ticks, and the abundance of *E. cervi* L1 statistically differed among the 4 main genotypes (Table 3).

**Table 3.** Test statistics of GLMM for effects of haplotype (2, 4, 5, 6) on tuberculosis infection, different abundances of parasites (*E. cervi*, tricostrongylid, ticks) and life traits (spleen mass and KFI) of red deer (excluding calves). All the models were fitted by year (2000-2005, $P > 0.05$ in every model). Degree of freedom refers to the maximum value in the model. n.s. not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

| Response variable ► | Tuberculosis (df=37) | | *E. cervi* (df=35) | | Ticks (df=37) | | Spleen mass (df=37) | | Body condition (df=37) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Explanatory variable▼ | *F* | *P* | *F* | *P* | *F* | *P* | *F* | *P* | *F* | *P* |
| Sex | 11.19 | ** | 51.67 | *** | 116.4 | *** | 1.94 | n.s | 23.21 | *** |
| Age | 4.93 | ** | 11.75 | *** | 25.60 | *** | 26.26 | *** | 18.39 | *** |
| Season | 0.01 | n.s. | 50.04 | *** | 0.01 | n.s | 273.9 | *** | 130.83 | *** |
| Body length | 11.93 | ** | 65.67 | *** | 2.44 | n.s | 147.4 | *** | 2.19 | n.s. |
| **HAPLOTYPE** | 8.17 | *** | 19.34 | *** | 7.85 | *** | 84.72 | *** | 3.52 | * |
| Season*Haplotype | 5.64 | ** | 15.42 | *** | 9.44 | *** | 3.33 | * | 21.81 | *** |

Overall, the results of paired comparisons (Table 4 displays the least square means, see also Fig. 2a) indicated that: (i) *CeelHap2* showed low infection rates of the *E. cervi* L1 infection; (ii) *CeelHap4* presented the highest abundance of infection by macroparasites (*E. cervi* L1 and ticks); (iii) *CeelHap5* showed low prevalence of TB, whereas *CeelHap6* showed high prevalence; (iv) Individuals with *CeelHap6* presented high prevalence of TB and low *E. cervi* scores, whereas individuals bearing *CeelHap5* presented a opposite trend.

Table. 4. This table shows the least square means ± S.E. of the respective models for tuberculosis infection status, parasitic infections (abundances of faecal *E. cervi* L1, faecal trichotrongylids eggs and ticks) and some life-traits (KFI and spleen) of red deer (excluding calves), which account for the partial effects of the other explanatory variables. Significant *p*-values of *T*-tests for paired comparisons between least squares means of the different haplotypes (involving the haplotype at the bottom, and the respective haplotype at the top right) are indicated in parenthesis.

| Variable | ***CeelHap2*** | ***CeelHap4*** | ***CeelHap5*** | ***CeelHap6*** |
|---|---|---|---|---|
| **Tuberculosis** (n=51) | -13.6±49.6 | -1.7±3.5(H5, CeelHap6) | -8.4±3.32(H4, H 6) | 2.3±3.17(H4, CeelHap5) |
| ***E. cervi*** (n=49) | -4.2±1.2(H4, CeelHap5) | -0.9±1.1(H2, CeelHap5, CeelHap6) | -1.5±1.1(H2 ,H4, CeelHap6) | -3.6±1.2( CeelHap4, CeelHap5) |
| **Ticks** (n=51) | -6.3±53.5 | 1.1±0.4(H5,H6) | -0.2±0.5(H4,H 6) | 0.6±0.4(H4, CeelHap5) |
| **Spleen** (n=53) | 6.0±0.1(H4, CeelHap5) | 5.7±0.1(H2, CeelHap5, CeelHap6) | 5.8±0.1(H2, H4, CeelHap6) | 6.0±0.1(H4, CeelHap5) |
| **KFI** (n=53) | 3.8±0.1(H5) | 3.6±0.1 | 3.5±0.1(H2, CeelHap6) | 3.7±0.1(H5) |

*Associations between MHC-II genotypes and life history traits*

After controlling by individual factors (including body size), spleen mass and body condition (KFI) differed between genotypes (Table 5 displays the least square means, see also Fig. 2b).

**Table 5.** Summary of infection susceptibility and life traits of red deer with the four more frequently found MCH haplotypes.

| | ***CeelHap2*** | ***CeelHap4*** | ***CeelHap5*** | ***CeelHap6*** |
|---|---|---|---|---|
| **Infections** | Low *E. cervi* | High *E. cervi* High ticks | High *E. cervi*, low ticks Low TB | High TB Low *E. cervi* |
| **Life traits** | High | Small spleen | Low | High |

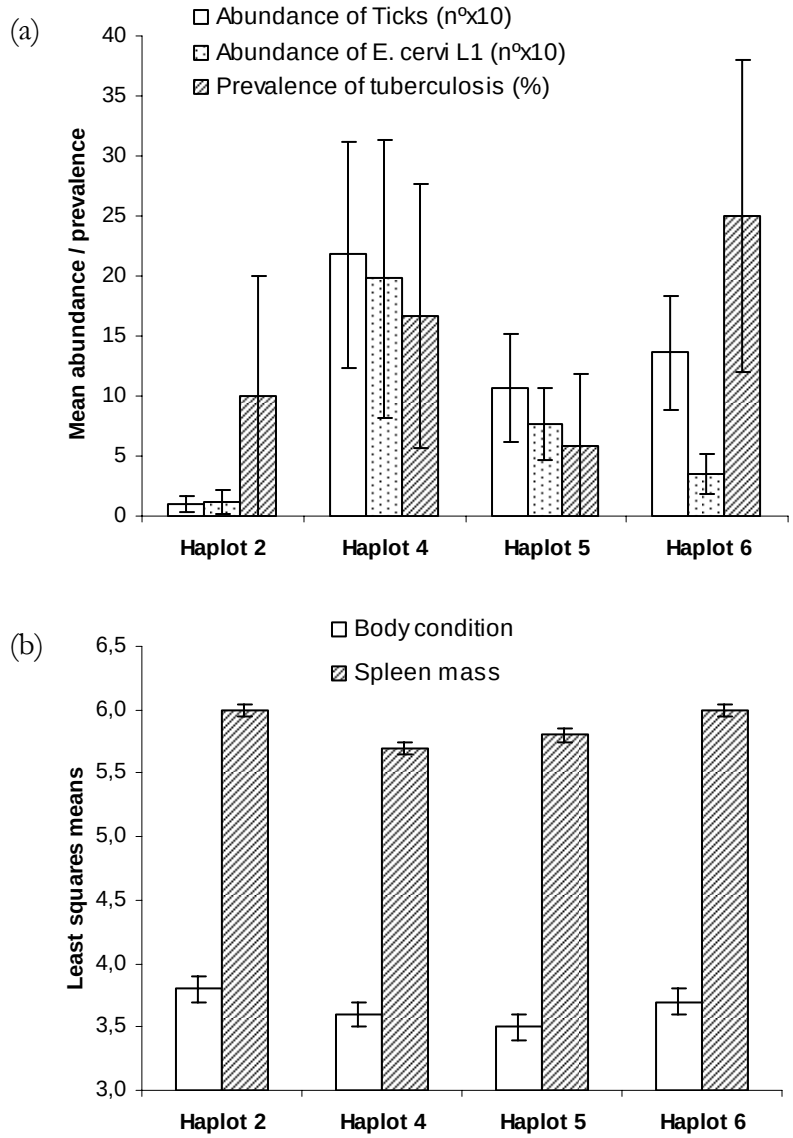


Figure 2. (a) Prevalence of tuberculosis and mean abundance of *E. cervi* L1 and ticks of red deer (excluding calves) from a population in Southwestern Spain with different MCH haplotype. It should be noted that the partial effects of the other explanatory variables are not accounted for (see least square means in Table 4). (b) Mean least squares for spleen mass and body condition (KFI) of red deer (excluding calves) from a population in Southwestern Spain with different MCH haplotype.

Paired comparisons between genotypes indicated that individuals with *CeelHap2* and *CeelHap6* had high relative spleen mass (statistically higher than individuals with *CeelHap4* and *CeelHap5*, *CeelHap4* individuals having the smallest relative spleen mass). Similarly, individuals with *CeelHap2* and *CeelHap6* showed high body condition scores (both statistically higher than individuals with *CeelHap5*).

*Associations between infections and life history traits*

A statistical negative relationship was found between KFI and *E. cervi* L1 counts ($F$ = 19.26, $p < 0.001$, parameter estimate = -0.01); tick counts ($F$ = 48.19, $p < 0.001$, parameter estimate = -0.01), and TB presence ($F$ = 14.00, $p < 0.001$, parameter estimate for the level absence = 0.24 considering a parameter estimate for the level presence = 0). Concerning spleen models, a statistical negative relationship was found between spleen mass and *E. cervi* L1 counts ($F$ = 58.41, $p < 0.001$, parameter estimate = -0.01); and tick counts ($F$ =7.36, $p < 0.01$, parameter estimate = -0.003). We did not evidenced statistical relationship between spleen mass and TB prevalence ($F$ = 0.03, $p < 0.88$, parameter estimate = 0.00). KFI and spleen mass statistically positively correlated ($F$ = 1062.86, $p < 0.01$, parameter estimate = 0.004).

## Discussion

We characterized individual genetic resistance to pathogens (looking at MHCII DRB-2 locus) while controlling epidemiological factors inherent to individuals in a single red deer population. Firstly, our results indicated functional significance of MHCII genes in regulating the defence of red deer against pathogens, although no single haplotype conferred general resistance against the complete range of pathogens. This may indicate that variation in the immune function associated to a single MHCII DRB locus differently underlies resistance against different pathogens. Secondly, the relationship between MHC genetics and life traits are the expected according to the relationships found between parasites and life traits, which confirms an important role to pathogens and host MHC in the viability of managed red deer populations.

*MHCII DRB-2 polymorphism and infections*

Each of the four most common haplotypes was characterized by specific resistance/susceptibility (according to infection rates) against certain pathogen/s (Tables 3 and 4, Fig. 1). Each MHC molecule has a different set of binding properties, and as a consequence, different peptides will be bound by these different molecules and each MHC allele will provide an infected individual with the ability to present a particular set of antigens (Hedrick 2002). Specific MHC alleles are especially effective at presenting antigens from a particular infection, and, hence, in combating specific pathogens (examples for single infections in a wide range of hosts: Langefors et al. 2001; Lohm et al. 2002, Paterson

et al. 1998; Harf and Sommer 2005; Meyer-Lucht and Sommer 2005; Schad et al. 2005). If MHCII molecules fail to bind or present some peptides derived from foreign proteins, an effective immune response cannot be achieved and further infection is likely. Therefore, our results can be explained by the variation in the pathogenesis of infection and in the specific host immune response against the considered pathogens.

Previous studies suggest that resistance of ungulates to intestinal nematodes might be associated with allelic variation at MHCII-DRB (MHCII DRB-2 locus in white tailed deer *Odocoilus virginianus*, Ditchkoff et al. 2005; in sheep, Schwaiger et al. 1995). Only indirect evidence suggests the involvement of DRB (or any MHC locus) in resistance against extrapulmonary lungworms in cervids (*Paralephastrongylus tenuis* in moose, Wilson et al. 2003). The importance of immunogenetics studies in relation to *E. cervi* resides in that it is wide spread and abundant in red deer across our study area (Vicente et al. 2006), it has demonstrated to interact with hosts body condition (Vicente et al. 2007a; b), and even it may prove a good immunological indicator in cervids (Gaudernack et el. 1984).

Similarly, Ditchkoff et al. (2005) evidenced resistance to ticks associated to MHCII DRB-2 in white tailed deer. Also host genetic factors (antigens of the bovine MHC also called bovine lymphocyte antigens, BoLA system) in cattle have been associated with tick resistance (Stear et al. 1984) and some BoLA class II alleles determine at least partly the susceptibility to tick infestation (Acosta-Rodríguez et al. 2005). Male sand lizards (*Lacerta agilis*) with a specific restriction fragment length polymorphism fragment in their MHC genotype are more resistant to *Ixodes ricinus* than are males lacking this fragment (Olsson et al. 2005). Genetics basis to control tick is important because they can cause severe direct impact on red deer and transmit a range of microparasitic infections of clinical relevance across our study area (e. g. Höfle et al. 2004; de la Fuente et al. 2004a, b).

We found that the risk of presenting TB statistically depended on the haplotype. Control of tuberculosis depends critically on the recognition and elimination of infected cells by sensitized CD4+ T cells. It is now well established that intracellular proteins can be also presented by MHC-II molecules (Chicz et al. 1993; Lechler et al. 1996), although the underlying processes are less clear (Dengjel et al. 2006). Crosstalk between MHC I and II pathways has been observed (Dengjel et al. 2005), which explain the existence of DRB protective haplotypes. CD4+ T cells, activated through MHC class II molecules, are essential for protection against tuberculosis by producing lymphokines such as IFN-g (Flory et al. 1992). For example, experimental studies in rodents showed that alveolar epithelial cells use the MHCII pathway to process and present mycobacterial antigens to

immune CD4+ T cells, serving as antigen-presenting cells during the early events of TB pathogenesis (Debbabi et al. 2005), which may determine the subsequent process. Population studies in humans have identified allelic associations between disease phenotypes and polymorphisms within the genes not only encoding class I (A,B,) but also class II (DR, DQ, DP) molecules of the MHCII (Papiha et al. 1987; Newport and Blackwell 1997; Ravikumar et al. 1999; Amirzargar et al. 2004). The predominant subset of lymphocytes that proliferated in response to in vitro stimulation with PPD was the CD4+ subset in white tailed deer (Waters et al. 2000), which is associated to the MHCII pathway. Also strong labelling of MHCII antigen in badger tubercles has been found in immunohistochemical studies in TB infected badgers, and bronchial epithelium expressed MHCII antigen strongly (Canfield et al. 2002). Our findings are important because TB progressively causes fatal wasting disease in red deer with very low rate of recovery, with high morbidity and mortality in our study area (Vicente et al. 2007c). In this sense, previous research in our study area suggests that some individuals may be genetically refractory (measured in neutral markers) to TB implantation or may display some degree of disease contempt (wild boar in our study area, Acevedo-Whitehouse et al. 2005; Naranjo et al. 2006a, b).

Our study system may well resemble the inherent complexity to natural systems since included parasites of different epidemiology and pathogenesis (macroparasites and microparasites, Nokes 1992, Maizels et al. 2004). We remark that no single haplotype conferred general resistance against the whole range of pathogens, which may indicate that variation in the immune function associated to a single MHC locus differently underlies resistance/susceptibility against different pathogens. Different alleles involved resistance/susceptibility to one class or group of parasites, either metazoan macroparasites (*E. cervi* and ticks) or tuberculosis. In particular, whereas *CeelHap5* showed low levels of TB and high abundance of *E. cervi* L1, the trend was the opposite in *CeelHap4* (Table 4). This may represent an immune trade-off the genetic basis to mount an appropriate immune response to different pathogens, as suggested for different MHCII DRB-2 variants, ticks and gastrointestinal nematodes in white tailed deer (Ditchkoff et al. 2005). We also found that specific haplotypes associated with high macroparasite infection, whereas other associated with low levels of them (Tables 4 and 5). Activation of immune response to suppress macroparasites may relay in part in similar mechanisms as they are extracellular pathogens and MHCII molecules are involved mainly in pathogen presentation of extracellular pathogens to CD4+ T-helper cells. Finally, it may be indicative of the

complexity of the immunogenetics relationships between hosts and pathogens that *CeelHap5* showed high levels of *E. cervi* L1, and low levels of ticks.

Population substructure (see discussion bellow) with subsequent increased contact within family groups (sharing a "family" genotype) could contribute to explain differences in infection rates rather than actual genetic differences (Blanchong et al. 2007). We found that haplotypes also related to indirectly transmitted (Vicente et al. 2006 in relation to *E. cervi* in watering areas), and to ubiquitous pathogens (apparently exposition did not differ between groups), which supports that differences related to genetics constitution.

*MHCII DRB-2 polymorphism and life history traits*

After controlling by individual factors (including body size), body condition and spleen mass statistically differed between MHCII DRB haplotypes (Table 5). To our knowledge this is the first time that specific relationships between MHC variations and life traits are reported in red deer. Interestingly, we found a statistical negative relationship between KFI and *E. cervi* L1 counts, tick counts, and TB presence; respectively. Also, after controlling by individual factors, *CeelHap2* and *CeelHap6* showed high KFI and relative spleen mass, with low levels of macroparasites (Table 5). These correlational results are consistent with increased parasitism or disease because due to individual genetic characteristics may lead to reduce fitness components. In white tailed deer there have been detected associations between genetic characteristics at Odvi-DRB and antler development and body mass, suggesting that they may associate with pathogen resistance in deer (Ditchkoff et al. 2001). How host genetics interaction with host nutrition and macroparasites? We hypothesize that if red deer with a particular configuration for DRB MHC locus have an immunologic advantage because of resistance to certain pathogens, MHC quality should be apparent in expression of condition-dependent traits, like KFI and spleen mass (Vicente et al. 2007a). Macroparasites have a negative impact on several red deer life-history traits such as nutritional status (Irvine et al. 2006; Vicente et al. 2007a, b), and therefore KFI varied as parasite did. A non-mutually exclusive possibility is that a particular MHC configuration also may confer more efficiency at obtaining food and greater metabolic efficiency, and subsequent good condition. It is well known that the nutritional status of the host can influence the rate of acquisition of immunity to parasitic and other infections in many animal species, including ruminants (e. g. Coop and Kyriazakis 1999; 2001; Strain and Stear 2001). Experimental approaches are needed to test the directionality of these relationships.

We evidenced statistical negative relationships between spleen mass and *E. cervi* L1 counts and ticks; respectively. Also, spleen mass and *E. cervi* L1 statistically and positively correlated. As previously reported, this is compatible with spleen mass being a condition-dependent trait (Vicente et al. 2007a), which is what one would expect considering that individuals in prime body condition would invest more (or more efficiently) in antiparasitic defences (Moller et al. 1998; Lochmiller and Deeremberg 2000). In summary, the spleen mass could reflect immune capacity in red deer, and individuals with larger spleens are apparently more capable of maintaining lower parasite levels. We stress that the interpretation of the relationships between MHC variants and life traits need to take into account variation in the relationships between MHC variants and specific parasite taxa.

*Management implications of host genetics and pathogens*

Red deer populations in South Central Spain display considerable variance in the prevalence and abundance of arthropods, helminths, and microbial pathogens that will each interact with the MHC. Some of them, like the widely spread TB, may impede current schemes of management of red deer populations (Gortázar et al. 2006; Vicente et al. 2007c). We should consider that schemes designed to maintain rare alleles (such as some MHC variants) or those providing a genetically advantage against diseases in wildlife could be operationally extremely difficult, we should have in mind that it could be as well counter-productive, for examples regarding the maintenance of genetic variation in the rest of the genome (Haig 1998; Hedrick and Miller 1994). Nevertheless, if it is possible to determine what alleles (especially those in risk to disappear) are advantageous, neutral, or detrimental; and planned translocations of individual carrying particular haplotypes could contribute to restore original genetic diversity and to increase population resistance to diseases. Also, if specific MHC alleles are known or suspected to be important for adaptation, then an approach to increase these alleles may be considered. A previous study on neutral loci in Iberian red deer suggests that differences among red deer populations in South Central Spain may rely on allele frequencies and/or the number of alleles (Martínez et al. 2002), which indicates that genetic goals in the management of Iberian deer populations have to avoid inbreeding to maintain potentially adaptive genetic variation.

Host–pathogen interactions are dynamic (Altizer et al. 2003) in such a way that 'protective' genotypes will not necessarily be so over longer time periods and geographical areas; hence, it would be necessary more studies before to attempt selection for particular genotypes. At this point, we suggest that immunogenetic data can help complement

management decisions in the context of genetic losses in such a managed ungulate. The combination with an experimental approach under standardized laboratory conditions is needed to prove the causal relationships behind correlations observed in the field. Also, complement research is needed in many other genes involved in shaping the immune repertoire, either solely or in conjunction with MHC (Acevedo-Whitehouse et al. 2007).

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# Capítulo 4

**Expresión diferencial de genes relacionados con respuesta inmune en ciervos infectados y no infectados, expuestos de forma natural a *Mycobacterium bovis***


**Expresión génica diferencial de genes implicados en la respuesta inmune e inflamatoria en linfonodos mesentéricos de ciervo ibérico (*Cervus elaphus hispanicus*) infectado de forma natural con *Mycobacterium bovis***

"Differential expression of inflammatory and immune response genes in mesenteric lymph nodes of Iberian red deer (*Cervus elaphus hispanicus*) naturally infected with *Mycobacterium bovis*"

## Expresión génica diferencial de genes implicados en la respuesta inmune e inflamatoria en linfonodos mesentéricos de ciervo ibérico infectado de forma natural con *Mycobacterium bovis*


### Resumen

Se dispone de muy poca información sobre la expresión de genes en infecciones micobacterianas naturales en especies de fauna salvaje. El ciervo ibérico puede actuar como reservorio de *Mycobacterium bovis* en España, aumentando así el riesgo de la tuberculosis bovina (TBb) en seres humanos y en la cabaña ganadera. En este trabajo se caracteriza la expresión diferencial de genes de la respuesta inmune e inflamatoria en linfonodos mesentéricos de ciervos infectados de forma natural con *M. bovis* usando la hibridación con un microarray. Estos resultados fueron validados con la determinación de concentraciones de proteínas séricas y/o de RT-PCR en tiempo real. De los 600 genes analizados en el microarray, 17 mostraron una expresión 1.7 veces mayor en ciervos infectados o no infectados ($Pp \leq 0.05$). Estos genes incluían proteínas de unión, IL-11R, bactenecina, CD62L, CD74, el desmogleina, IgA e IgM, lo que constituye nuevos hallazgos y sugiere nuevos mecanismos por los que *M. bovis* podría modular la respuesta inmunitaria e inflamatoria del hospedador. Estos resultados contribuyen al conocimiento básico de los mecanismos de patogénesis e inmunitarios de las infecciones micobacterianas naturales, pudiendo tener implicaciones importantes en el control de la TBb.

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Short communication

# Differential expression of inflammatory and immune response genes in mesenteric lymph nodes of Iberian red deer (*Cervus elaphus hispanicus*) naturally infected with *Mycobacterium bovis*

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**Abstract**

Little information is available about gene expression in natural mycobacterial infection of wildlife species. Iberian red deer can serve as reservoir of *Mycobacterium bovis* in Spain, thus increasing the risk of bovine tuberculosis (bTB) in humans and cattle. Herein, we characterized the differential expression of inflammatory and immune response genes in mesenteric lymph nodes of deer naturally infected with *M. bovis* using microarray hybridization. Results were validated by determination of serum protein concentrations and/or real-time RT-PCR. Of the 600 genes that were analyzed in the microarray, 17 genes displayed an expression fold change greater than 1.7 in infected or uninfected deer ($P \leqslant 0.05$). These genes included tight junction proteins, IL-11R, bactenecin, CD62L, CD74, desmoglein, IgA and IgM that constitute new findings and suggest new mechanisms by which *M. bovis* may modulate host inflammatory and immune responses. These results contribute to our basic understanding of the mechanisms of pathogenesis and immunity to natural mycobacterial infections and may have important implications for the control of bTB.


*Keywords:* Tuberculosis; Microarray; Deer; Wildlife; Lymph node; Genomics

## 1. Introduction

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* (*Mycobacterium tuberculosis* complex), is an established zoonotic disease, which affects cattle and wildlife worldwide [1]. The Iberian red deer (*Cervus elaphus hispanicus*) are infected with *M. bovis* and may be a reservoir of infection in some

*Abbreviations:* BTB; bovine tuberculosis; MLN; mesenteric lymph node

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regions of Spain [2,3]. In this species, mycobacteria apparently enter through the respiratory and digestive routes, subsequently causing lesions in several organs including lymph nodes, lung, liver and spleen. The digestive route is particularly important in deer in Mediterranean Spain [3].

Increasing evidence suggests that genetic and environmental factors contribute to the pathogenesis and differences in susceptibility of humans and mice to tuberculosis [4–7]. In European wild boar (*Sus scrofa*), which is a major reservoir of infection in South–Central Spain [2,3], genetic factors have been associated with resistance to bTB [8–10].

Recent studies using functional genomic analysis have provided data toward understanding the mechanisms involved in host–pathogen interactions and host cell responses to mycobacteria. However, only recently results have been reported about the immune response and the genes differentially expressed in naturally infected animal populations [10,13,14]. Analysis of genes differentially expressed in tissue biopsies collected from infected and uninfected animals exposed to mycobacteria in endemic areas would contribute to the understanding of the cellular response to natural mycobacterial exposure, thus providing information on genes that may correlate with resistance to tuberculosis and/or contribute to the development of protective responses in wildlife reservoirs of infection [1,9,10,13–15].

In the work reported herein, we characterized by microarray analysis, real-time reverse transcription (RT)-PCR and serum protein concentrations the inflammatory and immune response genes differentially expressed in mesenteric lymph nodes of Iberian red deer naturally infected with *M. bovis*. This is the first report of the global gene expression profile in deer and the first attempt to study inflammatory and immune response gene expression in deer naturally infected with *M. bovis*. Identification of genes differentially expressed in animals and tissues naturally infected with *M. bovis* contributes to our basic understanding of the mechanisms of pathogenesis, protective immunity and resistance to mycobacterial infections.

## 2. Materials and methods

### 2.1. Animals and sample preparation

Six adult (≥5-year old) Iberian red deer stags were hunter-harvested in a 900 ha hunting estate in the province of Ciudad Real, Castilla-La Mancha, Southwestern Spain (38°55′N; 0°36′E; 600–850 m above sea level). The major ruminant species present in the estate was Iberian red deer. Other wildlife present in the estate included European wild boar (*Sus scrofa*), as well as low numbers of introduced Barbary sheep (*Ammotragus lervia*) and mouflon (*Ovis ammon*). The range is fenced in order to restrict movement of wildlife. The mean prevalence of TB-compatible lesions among adult male red deer from Southern Spain is 17% [3]. The prevalence of bTB infections in the deer population in this estate, based on isolation of mycobacteria was 22% in adult stags ($N = 50$; unpublished data). Deer in this *M. bovis* endemic area freely mix and share common water and food supplies, which have increased animal interaction and exposure to the mycobacterium.

Hunter-harvested Iberian red deer was weighted, sexed and subjected to detailed necropsy [3]. The mesenteric lymph nodes were dissected and tissue fragments of approximately 2 $cm^3$ were prepared and stored at −80 °C for RNA extraction. The rest of the sample was used for culture and spoligotyping of mycobacteria [2].

*M. bovis* infection in deer was determined based on the presence of lesions characteristic of bTB and positive mycobacterial cultures and *M. bovis* spoligotypes [2,3]. Two deer had granulomatous lesions with positive *M. bovis* cultures in the MLN and four animals were negative for bTB characteristic lesions and mycobacterial cultures. Total RNA was isolated from biopsies of the MLN from these animals using RNeasy (Quiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNA was checked using the Experion™ Automated Electrophoresis System (Bio-Rad, Hercules, CA, USA) to evaluate the quality and integrity of RNA preparations. Three RNAs were partially degraded (<20% degradation) due to the fact that tissue biopsies were retrieved 2–12 h after animal hunting. RNA samples obtained from four uninfected deer and two *M. bovis*-infected animals were used for microarray hybridizations.

### 2.2. Microarray hybridization, scanning and analysis

Total RNA (1 μg) was labeled using the 3DNA Array900MPX kit with Alexa Fluor dyes (Genisphere, Hatfield, PA, USA), the supplied random and oligo-dT primes, Superscript II (Invitrogen, Carlsbad, CA, USA), the supplied formamide-based

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hybridization buffer and 24 × 60 mm LifterSlips slides (Erie Scientific, Portmouth, NH, USA) according to the manufacturer's (Genisphere) instructions. Ruminant immuno-inflammatory gene universal array (RIGUA) spotted with 75-base oligomers representing 600 ruminant genes were obtained from the University of Edinburgh (Scotland, UK). Hybridization signals were measured using a ScanArray Express (Perkin-Elmer, Boston, MA, USA) and the images were processed using GenePix Pro version 4.0 (Axon, Union City, CA, USA).

Three hybridizations were conducted, which included two hybridizations using total RNA from two individual, uninfected deer and one comparison using total RNA pooled from two uninfected individual animals. Pooling of total RNA from two uninfected animals was necessary due to a low yield of total RNA from these two animals. In each comparison, the control channels contained total RNA pooled from two different *M. bovis*-infected deer. RNAs from infected animals were pooled to minimize animal-to-animal variation in infected deer. Ratios were calculated as uninfected animals versus *M. bovis*-infected animals. Pre-processing of data was accomplished using *R*-project statistical environment (http://www.r-project.org) and Bioconductor (http://www.bioconductor.org) and the LIMMA package [16]. This included (1) removal of data points, where the signal was less than the background plus two standard deviations in both channels, (2) removal of data points, where signal was less than 200 RFU in both channels, (3) removal of poor quality spots flagged during image processing, (4) removal of spots with less than 50% valid biological and technical replicates, (5) log transformation of the background subtracted mean signal ratios and (6) normalization using global Lowess intensity-dependent normalization [17]. Normalized ratio values obtained for each probe were averaged across four biological replicates and three or more technical replicates. Significant differences were defined as $P$-values $\leqslant 0.05$ and displaying an expression fold change greater than 1.7-fold (log (base 2) ratio $\geqslant 0.8$). All the microarray data were deposited at the NCBI Gene Expression Omnibus (GEO) under the platform accession number GPL4456 and the series number GSE6083.

### 2.3. Real-time RT-PCR analysis

Due to the lack of deer sequence information in the databases, only five genes were analyzed for expression by real-time RT-PCR using primer sequences homologous to bovine or ovine genes (Table 1). For other genes, positive PCR results were not obtained. The expression of bactenecin (SMAP-29), IgG (a region identical between IgG1 and IgG2), interleukin (IL) 2 receptor (IL2-R) and casein kinase II (CK2) was quantified by real-time

Table 1
Primer sets and real-time PCR conditions used for this study

| Gene description | Genbank accession number | Upstream/downstream primer sequences (5′–3′) | PCR conditions[a] |
|---|---|---|---|
| *C. elaphus* β-actin | U62112 | BACT5 GAGAAGATGACCCAGATCA | 45 °C, 30 s/72 °C, 1 min |
| | | BACT3 GTTGCCGATGGTGATCACC | |
| *Ovis aries* bactenecin (SMAP-29) | L46854 | SMAP-29P5 ctacagggaggctgtgcttc | 45 °C, 30 s/72 °C, 1 min |
| | | SMAP-29P3 tgtcccacacactctttca | |
| *O. aries* immunoglobulin G1/G2 (IgG) | X69797/X70983 | IgG-P5 gaccactggactggaggaaa | 45 °C, 30 s/72 °C, 1 min |
| | | IgG-P3 actccacggcgatgtagtct | |
| *Bos taurus* interleukin 2 receptor | M20818 | IL2RP5 acgccatgttcaaggtcttc | 45 °C, 30 s/72 °C, 1 min |
| | | IL2RP3 gttctgcgcatctgtgtgtt | |
| *B. taurus* casein kinase II | X54962 | CK2P5 tttttccacggacatgacaa | 45 °C, 30 s/72 °C, 1 min |
| | | CK2P3 tcaggactgacaaggtgctg | |

[a]PCR conditions are shown as annealing/extension in RT-PCR analysis.

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RT-PCR in individual samples collected from 9–16 *M. bovis*-infected and 12–19 uninfected deer from the same study area but different from those used for the microarray analysis. Total RNA was extracted from mesenteric lymph nodes as described above for microarray analysis and used for real-time RT-PCR analysis. Real-time RT-PCR was performed with the RNA samples and gene-specific primers described above using the QuantiTec SYBR Green RT-PCR kit (Qiagen) and a Cepheid Smart Cycler II (Sunnyvale, CA, USA) following the manufacturer's recommendations. Amplification efficiencies were validated and normalized against deer (*C. elaphus*) β-actin (ACT) (GenBank accession number U62112) using the comparative Ct method. Real-time RT-PCR experiments were repeated at least three times with similar results.

To confirm the identity of analyzed genes, PCR fragments were purified using the PureLink™ PCR purification kit (Invitrogen, Carlsbad, CA, USA) and cloned into pGEM-T (Promega, Madison, WI, USA) for sequencing (Secugen SL, Madrid, Spain). Iberian red deer sequences for bactenecin (SMAP-29), IgG,IL2-R and CK2 were deposited in the Genbank under accession numbers EF053269–EF053272.

### 2.4. Determination of IgM, IgA and IgG serum levels

For the determination of IgM, IgA and IgG serum levels, 103 Iberian red deer samples (*M. bovis* infected, $n = 12$; uninfected, $n = 91$) were analyzed. Blood samples were collected into sterile tubes with and without anticoagulant (lithium heparin) and maintained at 4 °C until plasma and serum were separated after centrifugation and stored at −30 °C. The IgM, IgA and IgG serum levels were analyzed by turbidimetry using an automatic biochemical analyzer (A25, Biosystems, Barcelona, Spain) and manufacturer diagnostic reagents. Immunoglobulins in the sample were precipitated in the presence of goat anti-human IgM, IgA or IgG antibodies. The detection limit of the assay was 0.05 mg IgA and 10 mg IgM/IgG per dl serum. Serum protein levels were compared between TB+ and TB− wild boars by use of a one-tailed Student's *t*-test for samples with unequal variance ($P = 0.05$).

## 3. Results and discussion

The results reported here constitute the first analysis of differential gene expression in *M. bovis*-infected deer. The work with wild deer under natural conditions has the disadvantage of working with a limited number of individuals and on a single time point. However, it allows the analysis of gene expression in tissues naturally infected with *M. bovis*, such as the mesenteric lymph nodes, which are a target of mycobacterial infection in this species. The results of this analysis aided in defining a narrower set of genes to investigate in more detail the samples collected from a larger number of animals. The analysis of gene expression in tissues naturally infected with mycobacteria as opposed to experimentally infected bone marrow-derived macrophages and peripheral blood monocytes may be crucial to identify biomarkers and correlates of pathology and protection in tuberculosis [7]. This work, together with previous studies in European wild boar [9,10,13], provide information about gene expression in response to *M. bovis* in naturally infected wild ungulates.

Of the 600 ruminant inflammatory and immune response genes that were analyzed in the microarray, 157 showed ⩾1.2-fold changes in expression in infected or uninfected deer (Table S1, Appendix A). However, only 17 genes fulfilled the selection criteria of displaying an expression fold change greater than 1.7 with a *P*-value ⩽0.05 and were selected for further analysis (Table 2). We briefly summarize the function and possible role during mycobacterial infection of the proteins encoded by these genes.

- *Tight junction* (TJ) *protein 2* (*Z02*) *and occluding*: A TJ is a complex of integral and peripheral membrane proteins such as Z02 and occluding that interact strongly with the cytoskeleton to make the epithelial sheet between adjacent cells [18]. The expression of TJ proteins decreases after systemic inflammation and may be nitric oxide dependent [19]. As such, the expression of these genes could be down-regulated in *M. bovis*-infected animals.
- *Interleukin 2 and 11 receptors* (*IL-2R and IL-11R*): ILs such as IL-2 and IL-11 are involved in the protective response against mycobacterial infection. IL-2 is a Th1 cytokine that correlates with protection against bTB [14,15]. IL-11 inhibits macrophage cytokine production, stimulates B cell function, has protective mucosal effects and is induced by *M. tuberculosis* infection of human macrophages [11,20]. Therefore, down-regulation of IL-2R and IL-11R in infected deer may reflect a mechanism by which mycobacteria

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Table 2
Genes differentially expressed in *M. bovis*-infected and uninfected deer with log2 ratio changes ⩾0.8, ⩾50% good spots and $P \leqslant 0.05$

| Gene ID and description[a] | Ave log2 ratio[b] | Fold change[c] | % Good spots[d] | SD[e] | CV[f] | *P*-value[g] |
|---|---|---|---|---|---|---|
| *Down-regulated in infected deer* | | | | | | |
| AJ313187: Tight junction protein 2 (Z02) | 2.116 | 4.336 | 100 | 1.015 | 0.479 | 2.19E−05 |
| M20818: Interleukin 2 receptor (IL2-R, CD 25 or Tac antigen) | 1.272 | 2.415 | 56 | 1.288 | 1.026 | 0.000849 |
| X54962: Casein kinase II (CK2) | 1.160 | 2.234 | 89 | 1.183 | 1.032 | 0.000466 |
| AJ313182: Occluding | 1.046 | 2.065 | 56 | 1.700 | 1.744 | 0.054658 |
| L46854: Bactenecin (SMAP-29) | 1.036 | 2.051 | 78 | 1.287 | 2.406 | 0.010038 |
| L20311: $\mu$- and $\alpha$-IgM and IgA-associated J chain | 1.036 | 2.050 | 100 | 0.907 | 0.852 | 0.000172 |
| X59994: Immunoglobulin M (IgM) | 0.997 | 1.996 | 56 | 0.373 | 0.374 | 6.02E−08 |
| U32324: INTERLEUKIN 11 receptor (IL-11R) | 0.934 | 1.910 | 100 | 0.423 | 0.452 | 6.42E−07 |
| AF024645: Immunoglobulin A (IgA) | 0.860 | 1.815 | 100 | 0.819 | 0.956 | 0.000487 |
| *Up-regulated in infected deer* | | | | | | |
| AJ012589: Lysosomal integral membrane protein 1 (CD63) | −0.811 | −1.755 | 56 | 0.572 | 0.707 | 0.001855 |
| X62882: CD62L (L-selectin/LECAM-1) | −0.839 | −1.789 | 78 | 0.776 | 1.121 | 0.002323 |
| J318335: High affinity IgE receptor $\gamma$ subunit (FcERg) | −1.037 | −2.051 | 67 | 0.557 | 0.762 | 0.003688 |
| D83962: MHC II-associated invariant chain (CD74) | −1.059 | −2.084 | 89 | 0.850 | 0.802 | 0.001943 |
| X69797: Immunoglobulin G1 (IgG1) | −1.119 | −2.172 | 100 | 1.543 | 1.510 | 0.007093 |
| M58165: Desmoglein | −1.295 | −2.454 | 89 | 1.365 | 1.452 | 0.019836 |
| X70983: Immunoglobulin G2 (IgG2) | −1.407 | −2.652 | 100 | 1.448 | 1.240 | 0.000770 |
| AF109678: Small subunit ribosomal RNA | −1.437 | −2.707 | 56 | 1.508 | 1.088 | 0.000709 |

[a]Accession number from NCBI and description of the gene represented by the oligo in the spot/feature.
[b]Average log2 ratio is the Lowess print-tip normalized ratio (log2(635/532)) of background-corrected means averaged between valid biological replicates (uninfected/infected).
[c]Fold change of Lowess print-tip normalized log2 ratio of valid background-corrected means averaged between valid biological replicates (+, overexpressed and −, downregulated in uninfected animals).
[d]Percent of valid spots between and within slides included in ratio formulation.
[e]SD the standard deviation determined from the normalized average log2 ratio.
[f]CV is the coefficient of variation determined from the normalized average log2 ratio.
[g]*P*-value determined from the average log2 ratio using Bioconductor (http://www.bioconductor.org).

diminish host immune and protective responses against infection [21].

- *Casein kinase II* (*CK2*): CK2 is involved in the control of cell growth during infection [22], a process that is known to be affected by mycobacterial infection [23].
- *Bactenecin* (*SMAP-29*): Bactenecin is a cathelicidin antimicrobial peptide involved in humoral innate host defenses that interacts with the bacterial membranes, eventually leading to structural damage [24]. However, recent results suggest that binding of the cathelicidin peptides by the polysaccharides produced by lung pathogens can contribute to the impairment of peptide-based innate defenses of airway surface [25]. Additionally, mycobacteria may also reduce the expression of bactenecin to diminish host antimicrobial response in infected deer. Not surprisingly, deer bactenecin was closely related to ovine and bovine sequences (data not shown). However, this is the first report of bactenecin implication in mycobacterial infection.
- *Immunoglobulin* (*Ig*): Mycobacterial infections induce antibody production in rumminants [15,26,27]. However, the profile of Ig expression in *M. bovis*-infected animals is poorly understood [27]. Ig heavy and light chains were up-regulated in zebrafish and European wild boar infected with *M. marinum* and *M. bovis*, respectively [10,28]. However, serum determinations show elevated levels of IgG in uninfected boars when compared with *M. bovis*-infected animals [29]. The mechanism of differential expression of IgA, IgM, IgG1 and IgG2 in *M. bovis*-infected deer is unknown, but may reflect different stages during mycobacterial infection.
- *Lysosomal integral membrane protein 1* (*CD63*): Mycobacteria manipulate the endosomal system to arrest the maturation of their compartment and survive in the macrophage [23]. CD63 is a late

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endocytic marker of unknown function that is absent or non-abundant on mycobacterial phagosomes [30,31]. As in *M. bovis*-infected deer, the up-regulation of CD63 has been demonstrated in bovine macrophages infected with *M. avium* subsp. *paratuberculosis* [32] and in zebrafish infected with *M. marinum* [28], a finding that remains unexplained with current knowledge.

- *CD62L* (*L-selectin/LECAM-1*): Protection against tuberculosis depends upon the generation of CD4(+) T cell effectors capable of producing IFN-$\gamma$ and stimulating macrophage antimycobacterial function. Effector CD4(+) T cells are known to express a CD44(high)CD62L(low) surface phenotype [33]. Up-regulation of CD62L in *M. bovis*-infected cells may result in impaired macrophage antimycobacterial function at the site of infection.
- *Major histocompatibility complex class II (MHC-II)-associated invariant chain* (*CD74*): CD74, a non-polymorphic type II intracellular membrane glycoprotein, binds MHC-II glycoprotein and prevents peptides from binding to class II molecules, thus playing a regulatory role in MHC-II expression and antigen presentation [34]. The MHC-II antigen processing, which plays a central role in innate as well as acquired immunity, is one of the target mechanisms for immune evasion by mycobacteria [35]. Infection with mycobacteria inhibits MHC-II antigen processing in murine macrophages [35]. The up-regulation of CD74 in infected deer may represent an additional, previously unknown mechanism by which mycobacteria interfere with MHC-II antigen presentation by preventing peptide binding to MHC-II molecules.
- *Desmoglein*: Desmosomal cadherins such as desmogleins are the pathophysiologic targets of autoimmune or toxin-mediated disruption in the human diseases pemphigus and bullous impetigo, including its generalized form called staphylococcal scalded skin syndrome [36]. Although their role during tuberculosis has not been demonstrated, their up-regulation in *M. bovis*-infected deer may have pathologic implications.
- *Small subunit ribosomal RNA*: The small subunit ribosomal RNA was up-regulated in infected deer. *M. bovis* may affect protein metabolism by modulating the expression of ribosomal proteins as previously shown for infected European wild boar [10].

The analysis reported herein provides the basis for in-depth studies with fewer genes on a larger number of animals. The results of the microarray analysis were verified by real-time RT-PCR for bactenecin (SMAP-29), IgG, IL2-R and CK2 expression in individual samples of mesenteric lymph nodes of *M. bovis*-infected and uninfected deer, different from those used for the microarray analysis (Table 3). For other genes, positive PCR results were not obtained due to the lack of deer sequence information in the databases. The results were similar between real-time RT-PCR and microarray analyses. However, differences in the uninfected/infected ratio obtained by both methods may be due to differences in the sensitivity of the methods and/or the fact that ovine and bovine sequences were used in the microarray used to analyze deer RNA. At the protein level, IgM and IgG serum concentrations corroborated the results of the microarray analysis. IgG concentrations were

Table 3
Relative expression of selected genes in *M. bovis*-infected and uninfected deer

| MRNA levels (normalized Ct values) | | | |
|---|---|---|---|
| Bactenecin | IgG | CK2 | IL2-R |
| Uninfected deer (mean±SE) | | | |
| 2.140±1.939 | 0.309±0.147 | 0.013±0.007 | 0.020±0.005 |
| *M. bovis*-infected deer (mean±SE) | | | |
| 0.052±0.020 | 0.419±0.164 | 0.002±0.001 | 0.014±0.007 |
| Uninfected/*M. bovis*-infected deer (ratio) | | | |
| 41.2 | 0.7 | 5.2 | 1.4 |

The expression of bactenecin (SMAP-29), IgG, CK2 and IL2-R was quantified by real-time RT-PCR in mesenteric lymph nodes of *M. bovis*-infected ($N = 9$–16) and uninfected ($N = 12$–19) deer. Amplification efficiencies were validated and normalized against deer (*C. elaphus*) $\beta$-actin (ACT) (GenBank accession number U62112) and calculated by the $\Delta$Ct method. Real-time RT-PCR experiments were repeated at least three times with similar results.

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higher ($P<0.01$) in *M. bovis*-infected deer (Ave$\pm$SE; $1061\pm47$ mg/dl) than in uninfected animals ($581\pm131$ mg/dl), while IgM concentrations were higher ($P<0.05$) in uninfected ($44\pm6$ mg/dl) than in *M. bovis*-infected deer ($26\pm5$ mg/dl). IgA serum levels were not statistically different between infected ($17\pm4$ mg/dl) and uninfected ($14\pm2$ mg/dl) deer. The discrepancy between the results of the IgA microarray analysis and serum protein concentrations may be due to low affinity of anti-human antibodies for deer IgA.

As discussed above, the expression of some of the genes identified in this study, such as the IL-2R, IgGs, ribosomal RNA and CD63, have been reported previously to be affected by mycobacterial infection. However, other genes, including the IL-11R, bactenecin, CD62L, CD74, desmoglein, IgA, IgM, Z02 and occluding, constitute new findings and suggest new mechanisms by which *M. bovis* may modulate host inflammatory and immune responses.

Some inflammatory and immune-response genes reported to be affected by mycobacterial infections in previous studies in other species were not identified in the present work. This discrepancy may account for differences in the study system (isolated macrophages vs. lymph node tissue and/or species-specific differences; [10]) or technical reasons. The microarray used for the study reported herein was prepared for gene expression analysis in ruminants. However, some of the probes included in the array may not have sufficient homology to deer sequences to provide positive valid hybridization signals and therefore may result in underestimation of genes affected by *M. bovis* in deer.

The genes differentially expressed in *M. bovis*-infected deer likely reflect the effect of mycobacteria on host inflammatory and immune responses. Alternatively, these results may represent individual resistance and/or protective response to infection [6,9,10,12]. Uninfected deer were all adults in a *M. bovis* endemic area that mixed freely with infected animals and shared common water and food supplies, which have increased animal interaction and exposure to the mycobacterium. Some of the genes up-regulated in uninfected deer such as IgA, IgM and bactenecin may have important implications for vaccine development and characterization of protective immune response in deer and other ungulates.

In summary, some of the differentially expressed genes identified by this study have not been reported previously in mycobacteria-infected cells, thus expanding the existing information on the response of mammalian hosts to natural mycobacterial infections. These results propose new mechanisms by which mycobacteria affect host inflammatory and immune responses in naturally infected animals and suggest possible correlates of protective immunity to bTB in deer. These results may have important implications for future functional genomic and vaccine studies to aid in the control of bTB in wildlife.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dci.2007.05.001.

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# Appendix A. Tabla S1. Datos suplementarios

Supplementary material for Fernández de Mera et al.

Table S1. Genes with ≥ 50% good spots differentially expressed in *M. bovis*-infected and uninfected deer.

| Gene ID and Description[a] | Ave Log2 Ratio[b] | Fold Change[c] | % Good Spots[d] | SD[e] | CV[f] | P-value[g] |
|---|---|---|---|---|---|---|
| AJ313187: Z02 | 2,1165473 | 4,3365486 | 100 | 1,015 | 0,479 | 0,000022 |
| M20818: interleukin 2RA (CD 25 or Tac a | 1,2718945 | 2,4147846 | 56 | 1,288 | 1,026 | 0,000849 |
| X54962: casein kinase II | 1,1596878 | 2,2340908 | 89 | 1,183 | 1,032 | 0,000466 |
| AJ313182: occludin | 1,0464072 | 2,0653799 | 56 | 1,7 | 1,744 | 0,054658 |
| L46854: bactenecin (SMAP-29) | 1,0361277 | 2,0507160 | 78 | 1,287 | 2,406 | 0,010038 |
| L20311: J chain | 1,0357869 | 2,0502316 | 100 | 0,907 | 0,852 | 0,000172 |
| X59994: Immunoglobulin M | 0,9969873 | 1,9958278 | 56 | 0,373 | 0,374 | 0,000000 |
| U32324: interleukin 11RA | 0,9337036 | 1,9101734 | 100 | 0,423 | 0,452 | 0,000001 |
| AF024645: Immunoglobulin A | 0,8600808 | 1,8151400 | 100 | 0,819 | 0,956 | 0,000487 |
| NM_018402: interleukin 26 | 0,7156695 | 1,6422452 | 100 | 0,47 | 0,946 | 0,015624 |
| X97608: Cathelicidin -6 | 0,7079274 | 1,6334558 | 67 | 1,333 | 1,88 | 0,050291 |
| AF224266: interleukin 20 | 0,6992525 | 1,6236633 | 67 | 0,567 | 1 | 0,001629 |
| AB004061: Stat 2 | 0,6608464 | 1,5810099 | 78 | 0,702 | 1,042 | 0,001744 |
| AB011010: trappin 6 | 0,6505333 | 1,5697484 | 89 | 1,203 | 2,593 | 0,069446 |
| L34270: CD62P (P-selectin/PECAM) | 0,6422356 | 1,5607459 | 56 | 1,416 | 2,962 | 0,130226 |
| X97609: Cathelicidin -5 | 0,6409421 | 1,5593471 | 100 | 0,38 | 0,704 | 0,000731 |
| U38942: neuropeptide Y receptor 3 | 0,6373422 | 1,5554609 | 56 | 0,994 | 1,373 | 0,085701 |
| AF105370: beta-defensin 12,4,1,2,5 | 0,6172958 | 1,5339971 | 78 | 0,613 | 0,904 | 0,020899 |
| M57765: interleukin 11 | 0,5946227 | 1,5100776 | 100 | 0,223 | 0,375 | 0,001722 |
| U14944: ENaC-alpha | 0,5891831 | 1,5043947 | 89 | 0,384 | 0,763 | 0,001182 |
| AF290886: DC-SIGN | 0,5831889 | 1,4981571 | 78 | 0,565 | 0,958 | 0,001796 |
| NM_011414: SLPI | 0,5793917 | 1,4942190 | 89 | 0,692 | 1,688 | 0,019130 |
| M13973: protein kinase C | 0,5640252 | 1,4783883 | 100 | 0,3 | 0,488 | 0,000103 |
| TC133381: Complement Factor D | 0,5538633 | 1,4680115 | 100 | 1,013 | 1,829 | 0,046958 |
| AF010231: laminin A1 | 0,5366160 | 1,4505661 | 67 | 1,131 | 2,253 | 0,069140 |
| M14362: CD2 | 0,5344889 | 1,4484289 | 100 | 0,689 | 1,852 | 0,030645 |
| M89787: insulin-like growth factor 1 | 0,5317885 | 1,4457204 | 100 | 1,317 | 2,476 | 0,084070 |
| X54110: Immunoglobulin kappa chain | 0,5141518 | 1,4281542 | 67 | 0,649 | 0,997 | 0,020286 |
| D16680: c-kit | 0,5112174 | 1,4252524 | 67 | 0,666 | 1,327 | 0,008096 |
| AF254069: interleukin 21 | 0,4982537 | 1,4125028 | 89 | 0,59 | 1,178 | 0,005212 |
| AB039957: CD166 (ALCAM) | 0,4946195 | 1,4089491 | 67 | 0,693 | 11,361 | 0,114301 |
| AF068837: caspase 3 | 0,4786211 | 1,3934112 | 100 | 0,736 | 1,559 | 0,023765 |
| U60601: bactenecin 5 | 0,4722233 | 1,3872456 | 100 | 0,87 | 1,843 | 0,039880 |
| AF025303: insulin-like growth factor 1 | 0,4633570 | 1,3787462 | 56 | 1,209 | 3,368 | 0,239020 |
| E00135: interferon a3 | 0,4545287 | 1,3703351 | 78 | 0,827 | 2,047 | 0,040971 |
| Z48469: CD154 (CD40 ligand) | 0,4542968 | 1,3701148 | 67 | 0,868 | 1,736 | 0,059371 |
| U31628: interleukin 15RA | 0,4515390 | 1,3674982 | 100 | 0,555 | 1,225 | 0,010850 |
| M12807: CD4 | 0,4468372 | 1,3630488 | 67 | 0,677 | 1,45 | 0,032035 |
| AJ251357: MHC class II DYA | 0,4450659 | 1,3613763 | 56 | 0,957 | 2,334 | 0,082592 |
| AF270512: lutheran glycoprotein | 0,4412721 | 1,3578010 | 56 | 0,601 | 1,884 | 0,028399 |
| BM433105: Complement C1q alpha | 0,4393046 | 1,3559505 | 78 | 0,438 | 1,005 | 0,004062 |
| U63311: leukaemia inhibitory factor | 0,4305380 | 1,3477361 | 56 | 0,768 | 1,833 | 0,108379 |
| L46854: bactenecin (SMAP-29) | 0,4298294 | 1,3470742 | 78 | 1,287 | 2,406 | 0,150503 |
| AJ276489: Stat 3 | 0,4283013 | 1,3456482 | 67 | 0,681 | 2,948 | 0,051987 |
| X59068: interferon alpha-II Amy 49 | 0,4222415 | 1,3400079 | 100 | 0,744 | 1,738 | 0,033549 |
| AF113925: NOD1 | 0,4157125 | 1,3339573 | 89 | 0,462 | 1,855 | 0,050925 |
| AF162274: Complement C7 | 0,4116294 | 1,3301873 | 78 | 0,565 | 1,865 | 0,040313 |
| M25897: platelet factor 4 human | 0,4055457 | 1,3245899 | 56 | 0,686 | 1,677 | 0,064255 |
| AF079765: polycomb ECP1 | 0,4050970 | 1,3241779 | 67 | 0,504 | 20,16 | 0,335014 |
| AW652849: interleukin 10 receptor | 0,4042202 | 1,3233734 | 67 | 1,057 | 2,977 | 0,250990 |
| AF317803: erythroid associated factor A21 | 0,4034037 | 1,3226246 | 67 | 0,907 | 1,98 | 0,115339 |
| AY026859: SOCS3 (suppressor of cytokine | 0,3882777 | 1,3088300 | 67 | 0,61 | 1,658 | 0,130997 |
| AF245702: TLR7 | 0,3823598 | 1,3034722 | 78 | 0,482 | 1,272 | 0,019334 |
| AB055841: CD49d | 0,3762137 | 1,2979310 | 67 | 0,117 | 0,669 | 0,124075 |
| U59863: TRAF1 | 0,3736538 | 1,2956300 | 100 | 0,362 | 0,774 | 0,019443 |
| Y18205: FcERa | 0,3720600 | 1,2941995 | 67 | 0,522 | 1,434 | 0,026348 |
| M89789: insulin-like growth factor 2 | 0,3699675 | 1,2923237 | 89 | 0,503 | 1,74 | 0,036824 |
| U76873: MAP kinase | 0,3693372 | 1,2917593 | 56 | 1,051 | 2,695 | 0,185479 |
| M25897: CXCL4 | 0,3666177 | 1,2893265 | 56 | 0,749 | 2,035 | 0,084985 |
| NM_005283: CCXCR1 | 0,3631229 | 1,2862070 | 100 | 0 | 0 | 0,031054 |
| AF213396: fibroblast growth factor 10 | 0,3626400 | 1,2857766 | 89 | 0,428 | 1,166 | 0,020326 |
| U09861: HSP70 | 0,3566516 | 1,2804506 | 56 | 0,658 | 2,129 | 0,078124 |
| CB222525: CD19 | 0,3499005 | 1,2744727 | 78 | 0,332 | 1,328 | 0,042286 |
| AF109158: Duffy chemokine | 0,3469469 | 1,2718662 | 78 | 0,844 | 2,425 | 0,102815 |
| AW428020: interleukin 7 receptor | 0,3448402 | 1,2700103 | 89 | 0,436 | 1,29 | 0,084581 |
| Y09471: Cathelicidin -3 | 0,3410790 | 1,2667037 | 56 | 0,541 | 2,847 | 0,081365 |
| F14582: CD35 (CR1) | 0,3391961 | 1,2650515 | 56 | 0,278 | 1,645 | 0,078302 |
| TC139946: Complement C8b | 0,3387115 | 1,2646266 | 89 | 0,482 | 2,181 | 0,094662 |
| L41844: ICAM-3 | 0,3342884 | 1,2607554 | 78 | 0,577 | 1,702 | 0,056159 |
| U53484: thromboxane A2 receptor | 0,3247331 | 1,2524327 | 78 | 0,549 | 5,49 | 0,206034 |
| AF279437: interleukin 22 | 0,3131278 | 1,2423983 | 67 | 1,021 | 3,57 | 0,246627 |

Table S1. Cont

| | | | | | | |
|---|---|---|---|---|---|---|
| AF349458: CD49d (integrin alpha 4) | 0,3040675 | 1,2346204 | 89 | 0,493 | 1,638 | 0,162970 |
| AF207860: bradykinin receptor | 0,3035560 | 1,2341827 | 100 | 0,543 | 1,892 | 0,066439 |
| D16412: B cell receptor | 0,3008653 | 1,2318831 | 89 | 0,598 | 2,743 | 0,117438 |
| AF349462: integrin beta 3 | 0,3001702 | 1,2312896 | 67 | 0,571 | 5,387 | 0,140904 |
| AF214525: MHC class I LMP7 | 0,2982169 | 1,2296237 | 100 | 0,602 | 2 | 0,089144 |
| L31581: CCR7 (G protein-coupled receptor) | 0,2980183 | 1,2294545 | 78 | 0,509 | 3,242 | 0,118063 |
| U65979: interferon alpha/beta receptor | 0,2822548 | 1,2160941 | 100 | 0,479 | 2,207 | 0,086624 |
| TC129928: interleukin 17 | 0,2816781 | 1,2156080 | 56 | 0 | 0 | 0,307782 |
| U16261: interleukin 24 (MDA-7) | 0,2801662 | 1,2143348 | 67 | 0,929 | 3,117 | 0,233665 |
| AY040566: interleukin 22 binding protein | 0,2778884 | 1,2124191 | 100 | 0,206 | 1,364 | 0,047074 |
| U85589: interleukin 2R alpha chain | 0,2650914 | 1,2017121 | 67 | 0,259 | 2,123 | 0,130594 |
| M59818: GCSF receptor | 0,2590437 | 1,1966852 | 56 | 0,665 | 2,578 | 0,174251 |
| X60149: interleukin 2RB | 0,2590275 | 1,1966718 | 78 | 0,76 | 5,891 | 0,245992 |
| S63356: interleukin 9 | 0,2589436 | 1,1966022 | 56 | 0,515 | 2,164 | 0,116008 |
| Y13248: CXCR6 | 0,2589176 | 1,1965806 | 100 | 0,427 | 1,271 | 0,097625 |
| AF245219: DC-SIGNR | 0,2559262 | 1,1941021 | 89 | 0,537 | 2,131 | 0,121130 |
| U77845: TRIP | 0,2544895 | 1,1929136 | 89 | 0,349 | 1,369 | 0,062242 |
| BE480577: GMCSFRa/b (BARC 5BOV 5) | 0,2541097 | 1,1925995 | 56 | 0,492 | 2,016 | 0,252485 |
| L36232: transforming growth factor alpha | 0,2537522 | 1,1923041 | 100 | 0,553 | 4,127 | 0,203141 |
| X54183: macrophage scavenger receptor | 0,2468419 | 1,1866067 | 56 | 0,214 | 0,82 | 0,203480 |
| NM_010553: interleukin 18 receptor activated protein | 0,2427183 | 1,1832200 | 67 | 0,299 | 8,081 | 0,305215 |
| M15477: interferon beta1 | 0,2386643 | 1,1798997 | 56 | 0,483 | 4,391 | 0,064399 |
| Z28518: MHC class II DQA cosmid 39.1 | 0,2367960 | 1,1783727 | 56 | 0,676 | 2,889 | 0,270525 |
| AF110317: MHC class I TAP2 partial | 0,2295752 | 1,1724897 | 100 | 0,486 | 1,573 | 0,221134 |
| AF215981: CCR10 | 0,2196108 | 1,1644194 | 89 | 0,649 | 2,617 | 0,243394 |
| Y08133: mast cell protease-2 | 0,2195368 | 1,1643597 | 67 | 0,503 | 2,418 | 0,198128 |
| J04196: p21 Ras | 0,2190527 | 1,1639690 | 56 | 0 | 0 | 0,238751 |
| M15479: interferon beta3 | 0,2188676 | 1,1638197 | 89 | 1,04 | 4,749 | 0,362899 |
| AF110028: proteasome subunit LMP2 | 0,2182231 | 1,1632999 | 89 | 0,808 | 3,741 | 0,369072 |
| AF063109: carboxypeptidase E | 0,2153223 | 1,1609632 | 78 | 0,364 | 1,19 | 0,165094 |
| L04797: secretory component | 0,2018882 | 1,1502027 | 89 | 0,493 | 1,536 | 0,290504 |
| AJ313183: Z01 (tight junction protein 1) | 0,1999090 | 1,1486259 | 56 | 0,705 | 2,217 | 0,361483 |
| AF254067: interleukin 21R | 0,1961086 | 1,1456041 | 89 | 0,593 | 15,605 | 0,402615 |
| AB047030: beta-neuronatin 3 | 0,1926227 | 1,1428394 | 67 | 0,52 | 6,933 | 0,308470 |
| AF069053: SUG1 | 0,1851549 | 1,1369390 | 100 | 1,145 | 8,419 | 0,497455 |
| U62124: leptin receptor | 0,1851209 | 1,1369122 | 56 | 0,758 | 3,867 | 0,335000 |
| TC133725: Complement Factor B | 0,1820845 | 1,1345219 | 67 | 0,526 | 2,711 | 0,243203 |
| AF245704: TLR9 | 0,1815892 | 1,1341325 | 100 | 0,497 | 2,644 | 0,260354 |
| U10089: interleukin 7 | 0,1729635 | 1,1273719 | 56 | 0,823 | 4,378 | 0,423658 |
| AJ133642: prostaglandin D2 synthase | 0,1726629 | 1,1271370 | 78 | 0,752 | 4,132 | 0,408825 |
| AF310951: TLR2 | 0,1715992 | 1,1263063 | 78 | 0,447 | 4,912 | 0,304222 |
| AF170490: fibroblast growth factor receptor | 0,1714800 | 1,1262133 | 89 | 0,479 | 9,58 | 0,344306 |
| U68486: prostaglandin H synthase | 0,1711841 | 1,1259822 | 67 | 0,326 | 1,663 | 0,324342 |
| AB070717: interferon-gamma-inducible protein | 0,1690835 | 1,1243440 | 78 | 0,706 | 4,331 | 0,403034 |
| NM_005508: CCR4 | 0,1689571 | 1,1242455 | 100 | 0,35 | 1,598 | 0,214552 |
| X98240: CD13 | 0,1657657 | 1,1217613 | 78 | 0,46 | 460 | 0,373101 |
| E00136: interferon a4 | 0,1609322 | 1,1180093 | 78 | 0,506 | 3,329 | 0,321783 |
| S67956: monocyte chemoattractant protein | 0,1604512 | 1,1176366 | 56 | 0,21 | 2,625 | 0,493337 |
| AF000362: enteric prepro-beta-defensin | 0,1597885 | 1,1171233 | 67 | 0,933 | 3,029 | 0,565035 |
| Y15747: c-fos | 0,1586000 | 1,1162034 | 89 | 0,521 | 3,361 | 0,319664 |
| AF458059: interleukin 25 | 0,1574579 | 1,1153202 | 89 | 0,583 | 14,575 | 0,433224 |
| M84356: Immunoglobulin E | 0,1564372 | 1,1145314 | 89 | 0,881 | 5,372 | 0,504621 |
| S52657: insulin-like growth factor binding protein | 0,1554445 | 1,1137647 | 78 | 0 | 0 | 0,559794 |
| AY050252: MIP1 alpha | 0,1550106 | 1,1134298 | 56 | 0,262 | 1,016 | 0,289270 |
| AF180389: PrP | 0,1527695 | 1,1117015 | 89 | 0,403 | 2,634 | 0,264435 |
| AF228446: interferon regulatory factor | 0,1500318 | 1,1095940 | 56 | 0,425 | 3,269 | 0,313435 |
| AF196327: interferon tau | 0,1492421 | 1,1089867 | 100 | 0,567 | 3,831 | 0,403234 |
| U35038: interleukin 5 | 0,1441320 | 1,1050656 | 56 | 0,403 | 2,963 | 0,570035 |
| X52993: CD3 delta | 0,1415621 | 1,1030989 | 67 | 0,667 | 39,235 | 0,513459 |
| AJ291475: CD86 | 0,1415072 | 1,1030569 | 89 | 0,417 | 2,957 | 0,303748 |
| X59067: interferon alpha-1 Amy 124 | 0,1401288 | 1,1020035 | 100 | 0,866 | 1,942 | 0,679415 |
| AF141017: FcgN | 0,1380518 | 1,1004181 | 89 | 0,732 | 4,946 | 0,472233 |
| X72308: macrophage chemoattractant protein | 0,1365648 | 1,0992845 | 89 | 0,527 | 3,711 | 0,386897 |
| AF349461: CD29 | 0,1362806 | 1,0990679 | 100 | 0,662 | 4,868 | 0,476186 |
| M97935: Stat 1 | 0,1324807 | 1,0961770 | 89 | 0,377 | 1,545 | 0,388963 |
| BE693211: Complement C6 | 0,1292080 | 1,0936931 | 78 | 0,416 | 2,993 | 0,335259 |
| AB042274: CD6 | 0,1255350 | 1,0909122 | 89 | 0,403 | 10,605 | 0,424054 |
| J03599: prostaglandin G/H synthase | 0,1236718 | 1,0895042 | 67 | 0,495 | 3,367 | 0,388071 |
| D87918: macrophage-colony stimulating factor | 0,1234585 | 1,0893432 | 67 | 0,845 | 5,951 | 0,606271 |
| AF213380: fibroblast growth factor receptor | 0,1204285 | 1,0870577 | 89 | 0,637 | 4,977 | 0,506033 |
| U65978: interferon alpha/beta receptor | 0,1190204 | 1,0859972 | 89 | 0,789 | 2,3 | 0,702467 |
| AF004944: cyclooxygenase-2 | 0,1182540 | 1,0854204 | 67 | 0,371 | 7,894 | 0,435934 |
| AJ308426: interleukin 12R beta2 | 0,1165467 | 1,0841367 | 89 | 0,838 | 7,351 | 0,604661 |
| U75250: beta defensin 1 | 0,1141475 | 1,0823353 | 89 | 0,877 | 6,695 | 0,690769 |
| M13439: fibroblast growth factor - acid | 0,1136542 | 1,0819653 | 89 | 0,384 | 3,459 | 0,376804 |

Table S1. Cont

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|---|---|---|---|---|---|---|
| AB031323: matrilysin | 0,1096074 | 1,0789346 | 78 | 0,281 | 1,319 | 0,424250 |
| X98697: Complement Factor H | 0,1083079 | 1,0779632 | 78 | 0,468 | 3,714 | 0,459662 |
| U88540: TLR1 | 0,1068969 | 1,0769094 | 56 | 0,803 | 8,543 | 0,657926 |
| X52945: heparin-binding growth factor | 0,0933369 | 1,0668349 | 89 | 0,892 | 10,747 | 0,689893 |
| X14150: transforming growth factor-beta | 0,0925664 | 1,0662653 | 67 | 0,208 | 5,073 | 0,408170 |
| D50323: elafin | 0,0914168 | 1,0654159 | 56 | 0,561 | 3,281 | 0,619358 |
| AF144097: Mcl-1 partial | 0,0899696 | 1,0643477 | 100 | 0,313 | 2,675 | 0,540388 |
| AY124007: TLR3 | 0,0894395 | 1,0639568 | 100 | 0,525 | 5,899 | 0,676862 |
| AF383946: caspase 13A | 0,0894314 | 1,0639508 | 78 | 0,956 | 13,278 | 0,712695 |
| M36271: transforming growth factor-beta | 0,0885571 | 1,0633062 | 89 | 0,551 | 6,407 | 0,589629 |
| D84333: Complement C5 | 0,0796740 | 1,0567792 | 67 | 0,606 | 8,417 | 0,735366 |
| U57745: CD40 | 0,0796226 | 1,0567415 | 78 | 0,535 | 6,688 | 0,709887 |
| AB008156: Complement C8a | 0,0787335 | 1,0560905 | 89 | 0,356 | 7,12 | 0,622989 |
| S51402: CD3 eta | 0,0765921 | 1,0545241 | 67 | 0,294 | 1,246 | 0,642895 |
| Z49058: Complement Factor D | 0,0752918 | 1,0535741 | 56 | 1,228 | 3,601 | 0,755311 |
| AJ131185: histamine H2 receptor | 0,0718096 | 1,0510342 | 78 | 0,496 | 12,4 | 0,703523 |
| AB020984: Stat 4 | 0,0707842 | 1,0502874 | 78 | 0,552 | 5,935 | 0,654937 |
| AF132036: CD16 (FcgRIII) | 0,0699049 | 1,0496475 | 89 | 0,32 | 11,852 | 0,682464 |
| U87539: annexin 6 | 0,0689123 | 1,0489255 | 56 | 0,357 | 5,409 | 0,595019 |
| AF072807: interleukin 13 | 0,0673914 | 1,0478203 | 89 | 0,604 | 31,789 | 0,684792 |
| D16109: phospholipase A2 receptor | 0,0641472 | 1,0454667 | 100 | 0,547 | 8,415 | 0,739796 |
| M26809: nerve growth factor 3 | 0,0595233 | 1,0421213 | 67 | 0,558 | 27,9 | 0,736506 |
| AF092533: granulocyte colony-stimulating factor | 0,0588409 | 1,0416286 | 78 | 0,697 | 5,957 | 0,810120 |
| Z46236: keratinocyte growth factor | 0,0580796 | 1,0410790 | 89 | 0,498 | 9,396 | 0,740571 |
| Z29533: MHC class II DN A | 0,0576321 | 1,0407562 | 100 | 0,806 | 15,208 | 0,795600 |
| AF223942: iNOS | 0,0573010 | 1,0405173 | 100 | 0,862 | 4,585 | 0,802761 |
| Z14137: tumour necrosis factor-beta | 0,0542370 | 1,0383098 | 78 | 0,513 | 9,5 | 0,736951 |
| AF213398: c-met | 0,0501904 | 1,0354016 | 67 | 0,473 | 9,653 | 0,769552 |
| M98498: Ezrin | 0,0497445 | 1,0350816 | 67 | 0,549 | 8,318 | 0,786922 |
| Z12963: T cell receptor Cd | 0,0497379 | 1,0350769 | 78 | 0,528 | 21,12 | 0,778823 |
| AF288823: Complement C1s | 0,0469148 | 1,0330534 | 67 | 0,726 | 4,84 | 0,823203 |
| AF141313: CD14 | 0,0468390 | 1,0329991 | 67 | 0,856 | 11,889 | 0,856123 |
| AY124008: TLR9 | 0,0451415 | 1,0317844 | 89 | 0,294 | 4,523 | 0,774674 |
| AB011671: CD95 (fas) | 0,0373092 | 1,0261981 | 78 | 0,332 | 4,548 | 0,812326 |
| unsub: tumour necrosis factor receptor | 0,0359676 | 1,0252442 | 56 | 0,703 | 26,037 | 0,852785 |
| X60167: interleukin 1 alpha | 0,0357068 | 1,0250589 | 78 | 0,424 | 12,471 | 0,793782 |
| AF228059: CD55 | 0,0344764 | 1,0241850 | 78 | 0,413 | 7,121 | 0,842344 |
| U20418: CF transmembrane conductance receptor | 0,0333400 | 1,0233786 | 78 | 0,489 | 20,375 | 0,826315 |
| AF109677: MHC class II LMP7 | 0,0321305 | 1,0225210 | 89 | 1,419 | 15,767 | 0,939254 |
| M25648: oxytocin | 0,0320319 | 1,0224511 | 67 | 0,647 | 18,486 | 0,894356 |
| U28934: CX3CR1 | 0,0296399 | 1,0207573 | 67 | 0,813 | 38,714 | 0,893201 |
| U76739: nNOS | 0,0282879 | 1,0198012 | 78 | 0,489 | 27,167 | 0,861046 |
| M84747: interleukin 9R | 0,0273264 | 1,0191218 | 78 | 0,466 | 5,065 | 0,886103 |
| AF417637: annexin 8 | 0,0238155 | 1,0166446 | 89 | 0,632 | 18,588 | 0,890453 |
| X68149: CXCR5 | 0,0232723 | 1,0162619 | 78 | 0,663 | 10,694 | 0,901463 |
| Z24681: erythropoeitin | 0,0220732 | 1,0154176 | 67 | 0,654 | 34,421 | 0,903420 |
| AB055312: cathepsin D | 0,0219746 | 1,0153482 | 100 | 0,247 | 2,186 | 0,898763 |
| L42977: bactenecin 7 | 0,0207005 | 1,0144519 | 100 | 0,886 | 24,611 | 0,926681 |
| AF354057: Lymphotactin precursor (SCYA1) | 0,0183629 | 1,0128095 | 89 | 0,465 | 5 | 0,916507 |
| AJ007043: RANTES | 0,0174243 | 1,0121508 | 78 | 0,35 | 4,795 | 0,906227 |
| L06320: interferon alpha receptor | 0,0163398 | 1,0113903 | 67 | 0,875 | 17,157 | 0,937167 |
| NM_145751: TRAF4 | 0,0151858 | 1,0105816 | 78 | 0,752 | 10,592 | 0,927410 |
| NM_021258: interleukin 22 receptor | 0,0151618 | 1,0105648 | 78 | 0,327 | 3,442 | 0,915877 |
| M33304: MHC class II DQ A1 3 | 0,0096239 | 1,0066931 | 67 | 0,611 | 3,942 | 0,966105 |
| U88329: Mx-1 | 0,0094438 | 1,0065674 | 56 | 0,386 | 18,381 | 0,961504 |
| L03546: urokinase-type plasminogen activator | -0,0014380 | -1,0009972 | 100 | 0,459 | 5,961 | 0,992169 |
| Z28424: MHC class II DQB1 cosmid 39.1 | -0,0047868 | -1,0033235 | 100 | 0,869 | 86,9 | 0,982513 |
| M15477: interferon beta1 | -0,0056037 | -1,0038917 | 56 | 0,483 | 4,391 | 0,981826 |
| AX244221: CCR11 | -0,0074856 | -1,0052021 | 56 | 0,314 | 44,857 | 0,951468 |
| AF165719: Stat 5 | -0,0103439 | -1,0071956 | 89 | 0,386 | 7,018 | 0,942178 |
| AF215907: interleukin 18 binding protein | -0,0163883 | -1,0114242 | 89 | 0,475 | 27,941 | 0,911298 |
| BF075672: CD27 MARC 2BOV | -0,0182552 | -1,0127339 | 67 | 0,552 | 19,714 | 0,912198 |
| AF307971: CD62E (E-selectin/ELAM) | -0,0192835 | -1,0134560 | 78 | 0,509 | 4,072 | 0,918728 |
| M96651: interleukin 5 receptor | -0,0231794 | -1,0161965 | 67 | 0,425 | 2,237 | 0,904556 |
| AF287008: TREM 1 | -0,0260384 | -1,0182123 | 78 | 0,261 | 2,806 | 0,838190 |
| AF092740: CD152 | -0,0292490 | -1,0204808 | 67 | 0,935 | 66,786 | 0,903155 |
| AY029231: erythropoeitin receptor | -0,0303154 | -1,0212354 | 56 | 0,387 | 3 | 0,865001 |
| X75671: CD32 (FcgRII) | -0,0365878 | -1,0256850 | 78 | 0,469 | 2,727 | 0,848578 |
| NM_003855: interleukin 18 receptor | -0,0392743 | -1,0275968 | 67 | 0,38 | 8,837 | 0,766431 |
| AY040367: interleukin 19 | -0,0418726 | -1,0294492 | 78 | 0,476 | 8,815 | 0,842274 |
| Z12967: T cell receptor Cy4 | -0,0442481 | -1,0311456 | 100 | 0,79 | 9,753 | 0,848792 |
| TC143747: Complement C2 | -0,0445369 | -1,0313521 | 89 | 0,595 | 24,792 | 0,792162 |
| M21097: CD19 | -0,0472821 | -1,0333164 | 67 | 0,585 | 14,625 | 0,841225 |
| Y09950: CD80 | -0,0477968 | -1,0336851 | 67 | 0,284 | 2,869 | 0,702889 |
| AF149249: granulocyte chemotactic factor | -0,0482663 | -1,0340216 | 78 | 1,181 | 6,827 | 0,898487 |

Table S1. Cont

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|---|---|---|---|---|---|---|
| U19947: interleukin 8 receptor (CXCR1) | -0,0503875 | -1,0355430 | 67 | 0,536 | 12,465 | 0,768282 |
| AF327651: insulin-like growth factor binding protein | -0,0527510 | -1,0372409 | 67 | 0,446 | 5,868 | 0,756543 |
| L26547: CDK1 | -0,0616181 | -1,0436356 | 67 | 0,644 | 12,385 | 0,732137 |
| AF038131: CD21 (CR2) | -0,0648547 | -1,0459796 | 78 | 0,396 | 9,9 | 0,678884 |
| AY197339: insulin-like growth factor binding protein | -0,0685470 | -1,0486600 | 78 | 0 | 0 | 0,699614 |
| AF164518: bak | -0,0686024 | -1,0487003 | 67 | 0,709 | 8,542 | 0,714164 |
| AF272828: CD69 | -0,0727725 | -1,0517359 | 100 | 0,654 | 46,714 | 0,695942 |
| AF134814: ceruloplasmin | -0,0738614 | -1,0525300 | 67 | 0,631 | 8,195 | 0,674597 |
| M73245: interferon omega | -0,0753808 | -1,0536391 | 67 | 0,576 | 8,348 | 0,694548 |
| AF305562: peroxiredoxin 2 | -0,0788966 | -1,0562099 | 78 | 0,368 | 2,103 | 0,601501 |
| AF298809: calpain 3 | -0,0790984 | -1,0563577 | 89 | 0,399 | 5,466 | 0,554100 |
| TC142843: Complement C1s | -0,0791158 | -1,0563704 | 78 | 0,225 | 0,869 | 0,619729 |
| AJ313188: Z01 (tight junction protein 1) | -0,0811055 | -1,0578283 | 78 | 0,572 | 2,542 | 0,692531 |
| D14085: conglutinin | -0,0823540 | -1,0587441 | 89 | 0,451 | 3,027 | 0,528976 |
| X06367: a-lactalbumin | -0,0847789 | -1,0605252 | 100 | 0,33 | 1,602 | 0,585506 |
| AF004024: interleukin-12 p40 | -0,0860873 | -1,0614875 | 78 | 0,244 | 27,111 | 0,599253 |
| AF173557: interleukin 12p35 | -0,0863228 | -1,0616608 | 89 | 0,562 | 11,957 | 0,685027 |
| D76416: MHC class II DM A | -0,0881955 | -1,0630397 | 89 | 0,555 | 7,115 | 0,651062 |
| U33748: interleukin 2RG | -0,0912937 | -1,0653251 | 89 | 0,534 | 2,825 | 0,653066 |
| Z28422: MHC class II DQ B1/2 | -0,0913938 | -1,0653990 | 89 | 0,612 | 3,171 | 0,613883 |
| C94604: Complement C1r | -0,0924180 | -1,0661556 | 78 | 0,567 | 15,75 | 0,635763 |
| AJ278010: Prnpd dopple | -0,0928608 | -1,0664829 | 100 | 0,744 | 6,47 | 0,628283 |
| Z12968: CD3 zeta | -0,0972045 | -1,0696987 | 100 | 0,65 | 6,019 | 0,578015 |
| AF105370: beta-defensin 12,1,2,4 | -0,0980057 | -1,0702929 | 78 | 0,518 | 5,286 | 0,611836 |
| AF252548: galectin | -0,0999248 | -1,0717176 | 78 | 0,532 | 5,32 | 0,512700 |
| S78869: mucin | -0,1004377 | -1,0720987 | 67 | 0,383 | 19,15 | 0,539720 |
| AF071015: vascular endothelium growth factor | -0,1030918 | -1,0740728 | 89 | 0,631 | 5,584 | 0,564869 |
| L36136: fibroblast growth factor 2 | -0,1054072 | -1,0757980 | 67 | 0,427 | 1,817 | 0,574008 |
| L25886: CD49b | -0,1062292 | -1,0764111 | 78 | 0,386 | 2,169 | 0,489245 |
| X52994: CD3 gamma | -0,1077351 | -1,0775353 | 67 | 0,479 | 2,36 | 0,534974 |
| M55622: T cell receptor Ca VJC region | -0,1117335 | -1,0805258 | 78 | 0,735 | 2,732 | 0,620946 |
| U45984: CCR6 | -0,1120286 | -1,0807468 | 89 | 0,729 | 7,439 | 0,581321 |
| X66999: Complement C4 | -0,1144659 | -1,0825742 | 100 | 0,226 | 1,378 | 0,302262 |
| M81129: superoxide dismutase | -0,1171908 | -1,0846208 | 100 | 0,333 | 1,86 | 0,395144 |
| AF251146: alpha-tubulin | -0,1185797 | -1,0856655 | 89 | 0,369 | 3,101 | 0,338153 |
| U26024: pigpen | -0,1198762 | -1,0866416 | 78 | 0,58 | 4,874 | 0,495095 |
| U88879: TLR3 | -0,1210345 | -1,0875144 | 78 | 0,467 | 3,924 | 0,432865 |
| AF283892: NFkB | -0,1218005 | -1,0880919 | 56 | 0 | 0 | 0,700763 |
| AF282985: Flt3l (isoform 1) | -0,1249370 | -1,0904601 | 78 | 0,652 | 5,216 | 0,494243 |
| D17395: prostaglandin F2a receptor | -0,1279991 | -1,0927770 | 67 | 0,331 | 1,663 | 0,316175 |
| AF109199: chloride channel p64 | -0,1289512 | -1,0934985 | 100 | 0,353 | 2,736 | 0,473081 |
| M82802: annexin XI | -0,1299124 | -1,0942273 | 100 | 0,559 | 24,304 | 0,494105 |
| BG937885: CD59 | -0,1309320 | -1,0950009 | 78 | 0,622 | 4,859 | 0,459274 |
| AJ297965: integrin beta 3 | -0,1322270 | -1,0959842 | 100 | 0,501 | 2,227 | 0,444547 |
| AB000509: TRAF5 | -0,1342138 | -1,0974946 | 56 | 0,375 | 2,641 | 0,303528 |
| Y12420: peroxisome proliferator-activator | -0,1393386 | -1,1014000 | 56 | 0,331 | 2,527 | 0,558292 |
| Z13986: T cell receptor Cy5 | -0,1403168 | -1,1021471 | 78 | 0,576 | 4,8 | 0,406119 |
| Z28523: MHC class II DQB2 cosmid 9 | -0,1412669 | -1,1028732 | 56 | 0,949 | 4,721 | 0,545997 |
| AF210380: nexin | -0,1413969 | -1,1029726 | 67 | 0,296 | 1,109 | 0,329139 |
| AF081273: interleukin 4RA | -0,1415087 | -1,1030580 | 89 | 0,394 | 1,498 | 0,421346 |
| AF296673: Toll-like receptor 10 | -0,1423291 | -1,1036855 | 100 | 0,505 | 1,863 | 0,453577 |
| BE685522: interleukin 6 receptor | -0,1454845 | -1,1061020 | 67 | 0,411 | 2,957 | 0,306426 |
| AJ439062: integrin beta 6 | -0,1468736 | -1,1071676 | 67 | 0,408 | 2,935 | 0,293966 |
| AF241243: T-bet | -0,1469975 | -1,1072626 | 78 | 0,276 | 0,942 | 0,396183 |
| AF327650: insulin-like growth factor binding protein | -0,1471368 | -1,1073696 | 100 | 0,532 | 4,189 | 0,360661 |
| BM431251: Complement C5 | -0,1498041 | -1,1094188 | 89 | 0,636 | 4,609 | 0,385609 |
| U52221: melatonin-related receptor | -0,1517190 | -1,1108923 | 89 | 0,512 | 3,346 | 0,398177 |
| X56972: interleukin 1 beta | -0,1534449 | -1,1122221 | 100 | 0,619 | 1,799 | 0,476520 |
| AF213397: hepatocyte growth factor | -0,1571508 | -1,1150828 | 67 | 0,443 | 1,885 | 0,329043 |
| U97485: transforming growth factor-beta | -0,1594198 | -1,1168379 | 67 | 0,364 | 1,625 | 0,265748 |
| L36854: mucin-like glycoprotein (GLYCAM) | -0,1614294 | -1,1183947 | 67 | 0,769 | 5,093 | 0,449309 |
| M81233: CD18 | -0,1618270 | -1,1187030 | 100 | 0,355 | 2,191 | 0,217730 |
| BE750305: CD8 beta | -0,1683320 | -1,1237585 | 67 | 0,574 | 4,071 | 0,299093 |
| U16031: Stat 6 | -0,1691858 | -1,1244237 | 89 | 0,676 | 4,361 | 0,359329 |
| U17836: ribosomal protein P2 | -0,1695531 | -1,1247100 | 78 | 0,359 | 1,973 | 0,320793 |
| Z12966: T cell receptor Cy3 | -0,1709271 | -1,1257817 | 67 | 1,238 | 7,987 | 0,583451 |
| F14494: Complement C9 | -0,1741246 | -1,1282796 | 100 | 0,473 | 2,718 | 0,367204 |
| M64923: C10 (laminin receptor) | -0,1751473 | -1,1290796 | 78 | 0,481 | 28,294 | 0,400414 |
| M73983: MHC class II DR A | -0,1768757 | -1,1304332 | 67 | 0,461 | 2,918 | 0,435115 |
| AF329970: CD150 (SLAM) | -0,1777426 | -1,1311126 | 89 | 0,653 | 65,3 | 0,471504 |
| AJ132003: eotaxin | -0,1796636 | -1,1326197 | 100 | 0,449 | 2,494 | 0,384064 |
| AF228061: DAF | -0,1821906 | -1,1346054 | 89 | 0,538 | 2,989 | 0,314263 |
| TC135908: Complement C1q receptor | -0,1847450 | -1,1366160 | 89 | 0,512 | 1,369 | 0,429931 |
| M11507: CD71 (transferrin receptor) | -0,1851336 | -1,1369223 | 56 | 0,399 | 4,03 | 0,272156 |
| AB021662: CD34 | -0,1854558 | -1,1371762 | 100 | 0,336 | 1,077 | 0,289118 |

Table S1. Cont

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|---|---|---|---|---|---|---|
| J03143: interferon gamma receptor | -0,1868159 | -1,1382487 | 78 | 0,351 | 1,819 | 0,213545 |
| AB038383: Stat 6 | -0,1884674 | -1,1395525 | 67 | 0,295 | 1,372 | 0,329040 |
| AF233077: Flt-1 (VEGFR) | -0,1917157 | -1,1421211 | 67 | 0,494 | 2,823 | 0,222483 |
| BF601810: CD11c | -0,1925322 | -1,1427678 | 89 | 0,437 | 1,379 | 0,320327 |
| AF239241: fibroblast growth factor 7 | -0,1959769 | -1,1454996 | 56 | 0,3 | 1,515 | 0,131659 |
| AF284499: C5a receptor | -0,1970367 | -1,1463413 | 78 | 0,395 | 1,348 | 0,215157 |
| U26010: MHC class I antigen gene, alpha | -0,1975062 | -1,1467145 | 100 | 1,159 | 23,653 | 0,530906 |
| AF030379: Flt-4 | -0,2016193 | -1,1499884 | 89 | 0,472 | 4,538 | 0,225958 |
| AB099893: CXCR3 | -0,2061243 | -1,1535850 | 78 | 0,513 | 2,29 | 0,299874 |
| Z12969: CD3 epsilon | -0,2105409 | -1,1571219 | 67 | 0,522 | 1,657 | 0,245086 |
| AF399642: CXCR4 | -0,2126660 | -1,1588276 | 56 | 0,563 | 1,671 | 0,281367 |
| J03137: phosphoinositide-specific phosphatase | -0,2136232 | -1,1595967 | 100 | 0,493 | 1,409 | 0,270723 |
| AJ006722: CD1 D | -0,2142007 | -1,1600610 | 78 | 0,481 | 2,369 | 0,188724 |
| AF005380: NRAMP1 | -0,2183837 | -1,1634294 | 89 | 0,369 | 1,281 | 0,136957 |
| X59416: CD8 alpha | -0,2205399 | -1,1651695 | 100 | 0,785 | 3,204 | 0,269636 |
| AF149700: interleukin 15 | -0,2212956 | -1,1657801 | 67 | 0,419 | 1,1 | 0,276720 |
| X12497: interleukin 1 alpha | -0,2233108 | -1,1674096 | 78 | 0,35 | 0,994 | 0,134076 |
| AF008307: beta-defensin 4,2,1,12 | -0,2304960 | -1,1732383 | 67 | 0,495 | 2,152 | 0,252962 |
| M74782: interleukin 3 receptor A | -0,2323140 | -1,1747176 | 56 | 0,464 | 7,484 | 0,243240 |
| M18243: cyclooxygenase-1 | -0,2357033 | -1,1774806 | 78 | 0,284 | 11,833 | 0,360426 |
| AB055107: pBAM-2 adrenomedullin-1 | -0,2370238 | -1,1785588 | 100 | 0,844 | 3,336 | 0,278952 |
| Z27401: MHC class II DYB exon 3 | -0,2441263 | -1,1843753 | 100 | 0,698 | 2,861 | 0,313653 |
| AJ010711: perforin | -0,2456542 | -1,1856303 | 56 | 0,858 | 4,105 | 0,409458 |
| L03545: Urokinase receptor | -0,2461889 | -1,1860698 | 56 | 0,812 | 3,087 | 0,272255 |
| Af143722: CD23 | -0,2704065 | -1,2061477 | 67 | 0,468 | 1,272 | 0,094047 |
| X51689: macrophage scavenger receptor t | -0,2747963 | -1,2098232 | 67 | 0,601 | 1,287 | 0,245095 |
| AJ400864: CD45 | -0,2778193 | -1,2123610 | 67 | 0,558 | 2,067 | 0,094705 |
| AF461422: interleukin 23 receptor | -0,2797491 | -1,2139837 | 89 | 0,662 | 2,443 | 0,132371 |
| U03882: CCR2 | -0,2821954 | -1,2160440 | 89 | 0,83 | 3,074 | 0,211925 |
| AY077840: macrophage inflammatory protein | -0,2838224 | -1,2174161 | 78 | 0,405 | 0,938 | 0,087376 |
| AF310952: TLR4 | -0,2883202 | -1,2212175 | 67 | 0,493 | 1,833 | 0,066657 |
| Af266468: CCR3 | -0,2932285 | -1,2253794 | 78 | 0,652 | 2,38 | 0,244972 |
| X95876: CXCR3 | -0,2941369 | -1,2261512 | 67 | 0,564 | 1,945 | 0,116658 |
| AF019384: scavenger receptor class B | -0,3063447 | -1,2365707 | 78 | 0,424 | 1,804 | 0,052085 |
| AF099674: platelet activating factor receptor | -0,3072332 | -1,2373325 | 56 | 0,391 | 1,245 | 0,057226 |
| Z37506: FcG2R | -0,3081642 | -1,2381312 | 78 | 0,562 | 2,044 | 0,080779 |
| X60065: beta-2-gpI mRNA for beta-2-glycoprotein | -0,3096771 | -1,2394303 | 78 | 0,67 | 2,012 | 0,157795 |
| NM_003853: interleukin 18 receptor activation protein | -0,3114327 | -1,2409394 | 56 | 0,582 | 2,035 | 0,203474 |
| AF038130: Complement C3 | -0,3219817 | -1,2500464 | 78 | 0,315 | 1,265 | 0,023986 |
| Z11520: MHC class II DR B1 | -0,3227502 | -1,2507125 | 78 | 0,955 | 2,809 | 0,167882 |
| AF210382: annexin 5 | -0,3298747 | -1,2569042 | 78 | 0,533 | 1,62 | 0,044174 |
| AJ414557: IkB kinase-gamma | -0,3300089 | -1,2570211 | 78 | 0,661 | 2,021 | 0,086337 |
| AB005287: thrombospondin 1 | -0,3300576 | -1,2570636 | 67 | 0,876 | 2,647 | 0,251809 |
| L15344: interleukin 14 | -0,3331340 | -1,2597470 | 67 | 0,481 | 1,411 | 0,037894 |
| U90937: tumour necrosis factor receptor | -0,3362618 | -1,2624811 | 67 | 0,616 | 2,007 | 0,063715 |
| Y12815: CCBP2 (D6) | -0,3405323 | -1,2662237 | 67 | 0,518 | 2,977 | 0,109220 |
| Z49880: MHC class II DO B | -0,3406361 | -1,2663148 | 56 | 0 | 0 | 0,109980 |
| U21092: TRAF3 (CRAF1) | -0,3428375 | -1,2682485 | 67 | 0,357 | 0,892 | 0,016957 |
| AW353414: CD11b1 | -0,3484424 | -1,2731853 | 78 | 0,532 | 1,556 | 0,038822 |
| Z12965: T cell receptor Cy2 | -0,3494907 | -1,2741107 | 89 | 0,758 | 1,681 | 0,109840 |
| M59240: Complement Factor B | -0,3565091 | -1,2803241 | 78 | 0,325 | 0,7 | 0,021340 |
| AF092739: CD28 | -0,3595895 | -1,2830608 | 89 | 0,627 | 1,727 | 0,056131 |
| AF251147: vimentin | -0,3596835 | -1,2831444 | 100 | 0,564 | 2,051 | 0,047222 |
| AF349459: CD49e (integrin alpha 5) | -0,3658078 | -1,2886029 | 78 | 0,522 | 1,178 | 0,047853 |
| X94298: tyrosine kinase receptor Flk1 | -0,3660612 | -1,2888293 | 100 | 0,445 | 1,216 | 0,013387 |
| AF257235: eIF-4E | -0,3748347 | -1,2966910 | 67 | 0,991 | 2,423 | 0,141616 |
| X96540: thrombospondin 2 | -0,3756994 | -1,2974684 | 100 | 0,585 | 1,556 | 0,033291 |
| L13938: phospholipase C | -0,3799192 | -1,3012690 | 89 | 0,176 | 0,354 | 0,051837 |
| M15478: interferon beta2 | -0,3847455 | -1,3056294 | 89 | 0,535 | 1,023 | 0,051863 |
| U89874: stem cell factor (1) | -0,3874751 | -1,3081021 | 67 | 0,35 | 0,933 | 0,006358 |
| AF076633: pulmonary surfactant-associated protein | -0,3901697 | -1,3105476 | 78 | 0,547 | 1,413 | 0,018482 |
| L34208: prostaglandin synthase | -0,3909997 | -1,3113018 | 67 | 0,797 | 4,025 | 0,135805 |
| AF228445: interferon regulatory factor | -0,3991559 | -1,3187361 | 78 | 1,422 | 3,656 | 0,268458 |
| U57840: CCR5 | -0,4118385 | -1,3303801 | 78 | 0,378 | 0,892 | 0,006000 |
| AJ250464: CD44 | -0,4221649 | -1,3399367 | 100 | 0,696 | 1,326 | 0,053219 |
| D78178: annexin 4 | -0,4293908 | -1,3466648 | 56 | 0,444 | 0,8 | 0,017024 |
| : mptA-F-komplett ABC transporter | -0,4333650 | -1,3503796 | 78 | 0,724 | 1,68 | 0,036264 |
| AF000137: connective tissue growth factor | -0,4415417 | -1,3580548 | 78 | 0,449 | 1,035 | 0,002440 |
| AF178930: NOD2 | -0,4442737 | -1,3606290 | 78 | 0,412 | 2,368 | 0,107481 |
| AF257464: c-jun | -0,4489705 | -1,3650658 | 67 | 0,459 | 0,983 | 0,005525 |
| AF201926: eNOS | -0,4725595 | -1,3875690 | 89 | 0,579 | 1,204 | 0,009224 |
| Y11045: Myd-1 (a) | -0,4730009 | -1,3879935 | 78 | 1,128 | 2,605 | 0,134022 |
| Y00750: prostaglandin endoperoxide synthetase | -0,4732590 | -1,3882419 | 100 | 0,523 | 1,083 | 0,011017 |
| Z68501: c-myc | -0,5004380 | -1,4146430 | 100 | 0,494 | 1,202 | 0,009268 |
| M99367: AM-derived chemotactic factor | -0,5035137 | -1,4176620 | 67 | 0,383 | 0,764 | 0,014893 |

Table S1. Cont

| | | | | | | |
|---|---|---|---|---|---|---|
| U92569: bax-alpha | -0,5123968 | -1,4264180 | 78 | 0,679 | 2,135 | 0,044482 |
| AF119571: macrophage migration inhibition factor | -0,5132894 | -1,4273008 | 100 | 0,398 | 0,776 | 0,008199 |
| BF654844: CD11a (LFA-1 alpha chain) MAR | -0,5170119 | -1,4309883 | 78 | 0,5 | 1,002 | 0,035777 |
| X81705: p53 | -0,5332305 | -1,4471661 | 78 | 0,649 | 1,363 | 0,034676 |
| BE750928: interleukin 11 receptor | -0,5337302 | -1,4476674 | 56 | 0,643 | 1,419 | 0,045786 |
| AB005148: interleukin 1RA | -0,5723195 | -1,4869122 | 67 | 0,681 | 1,027 | 0,008858 |
| AF166488: interleukin 17 | -0,5787861 | -1,4935920 | 89 | 0,656 | 0,91 | 0,025523 |
| AF140667: stromal-derived factor | -0,6024389 | -1,5182810 | 78 | 0,386 | 0,643 | 0,000205 |
| AF074402: interleukin 13RA | -0,6269478 | -1,5442944 | 67 | 0,638 | 1,981 | 0,063168 |
| AF272837: glyceraldehyde 3 phosphate dehydrogenase | -0,6337900 | -1,5516359 | 89 | 0,437 | 0,771 | 0,000201 |
| E00134: interferon a1 | -0,6527473 | -1,5721591 | 78 | 0,421 | 0,646 | 0,000345 |
| S77394: insulin-like growth factor-binding protein | -0,6613082 | -1,5815161 | 100 | 0,518 | 0,935 | 0,000707 |
| Z12964: T cell receptor Cy1 | -0,6670001 | -1,5877680 | 67 | 0,444 | 0,675 | 0,000206 |
| Z36890: CD1 B (A25) | -0,7008676 | -1,6254820 | 89 | 0,663 | 0,927 | 0,000822 |
| TC135848: perforin | -0,7057366 | -1,6309772 | 56 | 0,484 | 0,75 | 0,008941 |
| U95814: GRO | -0,7238125 | -1,6515406 | 56 | 0,486 | 0,585 | 0,000406 |
| AJ012589: CD63 | -0,8113255 | -1,7548229 | 56 | 0,572 | 0,707 | 0,001855 |
| X62882: CD62L (L-selectin/LECAM) | -0,8389047 | -1,7886917 | 78 | 0,776 | 1,121 | 0,002323 |
| AJ318335: FcERg | -1,0366877 | -2,0515121 | 67 | 0,557 | 0,762 | 0,003688 |
| D83962: CD74 invariant chain | -1,0595428 | -2,0842709 | 89 | 0,85 | 0,802 | 0,001943 |
| X69797: Immunoglobulin G1 | -1,1192732 | -2,1723750 | 100 | 1,543 | 1,51 | 0,007093 |
| M58165: desmoglein | -1,2949276 | -2,4536469 | 89 | 1,365 | 1,452 | 0,019836 |
| X70983: Immunoglobulin G2 | -1,4071122 | -2,6520577 | 100 | 1,448 | 1,24 | 0,000770 |
| AF109678: small subunit ribosomal RNA | -1,4369299 | -2,7074409 | 56 | 1,508 | 1,088 | 0,000709 |

[a]Accession number from NCBI and description of the gene represented by the oligo in the spot/feature.

[b]Average log2 ratio is the Lowess print-tip normalized ratio (log2(635/532)) of background-corrected means averaged between valid biological replicates (Uninfected/Infected).

[c]Fold change of Lowess print-tip normalized log2 ratio of valid background-corrected means averaged between valid biological replicates (Uninfected/Infected).

[d]Percent of valid spots between and within slides included in ratio formulation.

[e]SD is the standard deviation determined from the normalized average log2 ratio.

[f]CV is the coefficient of variation determined from the normalized average log2 ratio.

[g]P-value determined from the average log2 ratio using Bioconductor (http://www.bioconductor.org).

# Síntesis y Conclusiones

# Síntesis

En el presente apartado se exponen de manera sintética las principales conclusiones del trabajo de tesis, con especial atención a los factores implicados en la respuesta inmunitaria en el ciervo ibérico.

## Capítulo 1.- Factores que intervienen en la respuesta dérmica a la inyección de antígenos

Los experimentos que componen el primer capítulo de esta tesis han permitido conocer que la respuesta in vivo al mitógeno fitohemaglutinina y a los derivados purificados de proteínas micobacterianas en ciervos ibéricos depende principalmente de dos factores, el sexo y la condición física. En cambio, no se han detectado diferencias importantes en función de la edad.

Los machos de ciervo tienden a mostrar respuestas de incremento del pliegue cutáneo mayores que las hembras. Esta diferencia resulta especialmente marcada en condiciones de granja, presumiblemente porque la limitación de recursos tróficos es mucho mayor en las poblaciones silvestres que en cautividad. La mayor respuesta a los antígenos por parte de los machos ha sido comprobada en dos experimentos distintos (trabajos 2 y 3). El otro factor que influye fuertemente en la capacidad del ciervo para responder a las pruebas de intradermorreacción es su condición física. Esto se ha puesto de manifiesto en el trabajo 1, donde se comprobó que las hembras de mayor peso (a igualdad de otros factores) respondían mejor a la fitohemaglutinina que las hembras más ligeras. Este hallazgo se ha confirmado en el trabajo 3, al observar que los ciervos salvajes, presumiblemente en peor condición física, responden peor a los antígenos testados que sus homólogos de granja, mejor alimentados. En cuanto a la ausencia de efecto de la edad comprobada en el trabajo 2, es importante hacer constar que no han sido testados gabatos. Esta clase de edad podría tener una capacidad de respuesta diferente a la de los ciervos de más edad. Los gabatos normalmente no son objeto de testaje intradérmico hasta que alcanzan su primer invierno de vida, por lo que no fueron incluidos en este estudio.

En el plano más aplicado, estos trabajos contribuyen a la futura puesta a punto de criterios para la realización de pruebas de intradermorreacción en ciervos en España. Se trata de una herramienta necesaria en el marco de traslados de fauna silvestre y de la

producción en granjas cinegéticas. En el plano más teórico, se aporta información científica que respalda la hipótesis de que la capacidad de respuesta inmune en el ciervo ibérico está condicionada por su condición física, particularmente por su estado nutricional (Vicente et al. 2007 a y b)[1,2].

**Capítulo 2.- Relación entre condición nutricional y capacidad de respuesta antiparasitaria**

El experimento detallado en el segundo capítulo de esta tesis ha permitido constatar que la excreción fecal de larvas 1 del nematodo protostrongílido *Elaphostrongylus cervi* es mayor en ciervos que se encuentran en peor condición nutricional.

En igualdad de otros factores fisiológicos y ambientales, las ciervas que disponen de aporte suplementario de alimento presentan niveles de excreción de larvas sensiblemente inferiores a los que presentan las ciervas no suplementadas. Estos resultados coinciden con hallazgos anteriores de nuestro grupo, basados en necropsias. En concreto, se conocía que el tamaño del bazo (un indicador de la capacidad inmunitaria, Corbin et al. 2007)[3] varía de forma inversamente proporcional a los niveles de excreción de *E. cervi* (Vicente et al. 2007 a)[1], y que el tamaño del bazo y la excreción de *E. cervi* dependen del nivel de engrasamiento renal, un indicador de condición nutricional (Vicente et al. 2007 a)[1]. Una novedad importante es el carácter no invasivo de la técnica de estudio aplicada en esta tesis.

De forma práctica, los resultados obtenidos permiten utilizar los niveles de excreción de *E. cervi* (y por extensión posiblemente de otros parásitos en hospedadores similares) como indicador de condición en poblaciones silvestres. Esta utilidad de los análisis parasitológicos debe combinarse, en su caso, con otros indicadores del estado de una población de fauna silvestre (Gortázar et al. 2006)[4]. Desde un punto de vista evolutivo, el experimento del capítulo 2 apoya, igual que el capítulo 1, la hipótesis de que la capacidad de respuesta inmune en el ciervo ibérico está condicionada por su condición física, particularmente por su estado nutricional (Vicente et al. 2007 a y b)[1,2].

**Capítulo 3.- El factor genético: Polimorfismo del MHC II en ciervo ibérico**

Los estudios sobre polimorfismo del complejo mayor de histocompatibilidad de clase II en una población aislada de ciervo ibérico, gestionada con fines cinegéticos, han permitido constatar una variabilidad genética limitada y en regresión, dentro de la cual unos pocos genotipos aparecen con frecuencia muy superior a la esperada. Algunos de estos

genotipos dominantes resultaron estar asociados a mayores o menores prevalencias de tuberculosis o de parasitosis.

Además, los resultados obtenidos en el trabajo 6 demuestran que el nivel de infección de una población de ciervo por un determinado patógeno, por ejemplo *Mycobacterium bovis* o *Elaphostrongylus cervi*, puede variar sensiblemente en función de las características genéticas de dicha población, y particularmente de los genes del MHC II. El genotipo, por tanto, debe considerarse como un factor más que modula la relación patógeno-hospedador en el ciervo ibérico. Estos resultados coinciden en cierta medida con hallazgos previos en otras especies, como por ejemplo el jabalí (Acevedo-Whitehouse et al. 2005)[5].

Los resultados del capítulo 3 tienen implicaciones prácticas en gestión cinegética, ya que ponen de manifiesto que la variabilidad genética es un factor importante en la relación patógeno-hospedador, y puede encontrarse limitada o incluso empeorar en poblaciones valladas como las que abundan en España. Los resultados también tienen aplicación potencial en la selección a nivel de granjas y en la elección de animales en el marco de traslados, si bien es importante considerar aspectos éticos y de conservación de la biodiversidad. Desde el punto de vista teórico, el hallazgo de que determinados tipos de MHC II están relacionados no sólo con la probabilidad de infección por *M. bovis* o por parásitos, sino también con la condición física (engrasamiento renal) y la capacidad inmunitaria (tamaño del bazo), completa el círculo de los resultados de los primeros dos capítulos: la capacidad de respuesta inmunitaria estaría mediada (en el ciervo) al menos por los factores “sexo”, “condición física” y “genotipo”.

**Capítulo 4.- Expresión diferencial de genes relacionados con respuesta inmune en ciervos infectados y no infectados, expuestos de forma natural a *Mycobacterium bovis***

El análisis de RNA extraído de linfonodos mesentéricos de ciervos ibéricos ha detectado diferencias en la expresión de numerosos genes, de entre los 600 genes testados e implicados en la respuesta inmunitaria o inflamatoria en rumiantes. Entre los genes identificados, varios tienen relación directa con los mecanismos de respuesta inmunitaria y el complejo mayor de histocompatibilidad.

La expresión del gen del receptor para la interleucina 2 se encuentra inhibida en ciervos infectados con *M. bovis*, lo que sugiere que podría tratarse de un mecanismo por el que las micobacterias disminuyen la capacidad de respuesta inmunitaria del hospedador.

También el gen SMAP-29 que expresa la bactenecina, un péptido antimicrobiano que participa en la respuesta humoral innata, se encuentra inhibido en los ciervos infectados. Igualmente, la expresión de los genes de las inmunoglobulinas A, M y G varió entre ciervos infectados y no infectados, estando sobreexpresadas las de tipo G e inhibidas las de tipo A y M. La sobreexpresión del gen CD62L en ciervos infectados sugiere capacidad de *M. bovis* para alterar la respuesta inmunitaria, en este caso afectando a la función antimicrobiana de los macrófagos. Finalmente, se ha detectado en ciervos infectados por *M. bovis* la sobre expresión del gen de la cadena no variable del MHC-II (CD74). Esta glicoproteina intracelular no polimórfica evita la unión entre los péptidos y las moléculas de clase II, regulando por tanto la presentación de antígenos por parte de MHC-II. Esto podría constituir un mecanismo, hasta ahora desconocido, de las micobacterias para evitar la respuesta inmunitaria del hospedador.

En el aspecto aplicado, los resultados del capítulo 4 podrían resultar de interés para la caracterización de la respuesta inmunitaria protectora en ciervo y otros rumiantes, así como para el desarrollo de vacunas. En el aspecto teórico, los resultados expuestos sugieren que a los factores ya descritos que modulan la capacidad de respuesta del hospedador (sexo, condición física y genotipo), cabe añadir las características del agente patógeno, ya que al menos *M. bovis* es capaz de interferir algunos de los mecanismos de la respuesta inmunitaria del ciervo. Estos resultados, junto con los hallazgos recientes en jabalí (Naranjo et al. 2006 y 2007)[6,7] contribuyen al conocimiento de la expresión génica diferencial en respuesta a la tuberculosis en especies de fauna salvaje que podrían actuar como reservorio de tuberculosis.

[1]Vicente J, Pérez-Rodríguez L, Gortázar C. 2007a. Sex, age, spleen size and kidney fat of red deer relative to infection intesnities of the lungworm *Elaphostrongylus cervi*. Naturwissenschaften 94: 581-587.

[2]Vicente J, Höfle U, Fernández-de-Mera IG, Gortázar C. 2007b. The importance of parasite life history and host density in predicting the impact of infections in red deer. Oecologia 152: 655-664.

[3]Corbin E, Vicente J, Martín-Hernando MP, Acevedo P, Pérez-Rodríguez L, Gortázar C. Spleen mass as a measure of immune strenght in mammals. Mammal Review, en prensa.

[4]Gortázar C, Acevedo P, Ruiz-Fons F, Vicente J. 2006. Disease risks and overabundance of game species. European Journal of Wildlife Research 52: 81-87.

[5]Acevedo-Whitehouse K., Vicente J, Gortázar C, Höfle U, Fernández-de-Mera IG, Amos W. 2005. Genetic resistance to bovine tuberculosis in the Iberian wild boar. Molecular Ecology 14: 3209–3217.

[6]Naranjo V, Ayoubi P, Vicente J, Ruiz-Fons F, Gortázar C, Kocan KM, de la Fuente J. 2006. Characterization of selected genes upregulated in non-tuberculous European wild boar as possible correlates of resistance to *Mycobacterium bovis* infection. Veterinary Microbiology 116: 224–231.

[7]Naranjo V, Villar M, Martín-Hernando MP, Vidal D, Höfle U, Gortázar C, Kocan KM, Vázquez J, de la Fuente J. 2007. Proteomic and transcriptomic analyses of differential stress/inflammatory responses in mandibular lymph nodes and oropharyngeal tonsils of European wild boars naturally infected with *Mycobacterium bovis*. Proteomics 7: 220-231

# Conclusiones

1. Siguiendo las pautas descritas en esta tesis, el uso del mitógeno fitohemaglutinina, como medida independiente de la capacidad de respuesta inmunitaria, es adecuado para su empleo en ciervos.

2. El sexo y la condición física son dos factores que afectan a la intensidad de la respuesta del ciervo a la prueba de intradermorreacción frente a la fitohemaglutinina. Así, los machos muestran una mayor capacidad de respuesta que las hembras, y los animales en mejor condición nutricional muestran una mayor capacidad de respuesta que aquellos en peor condición.

3. La condición física, y en particular el estado nutricional del ciervo, puede influir en su capacidad de respuesta frente al parásito *Elaphostrongylus cervi*.

4. En poblaciones aisladas de ciervo ibérico, la pérdida de variabilidad en genes relacionados con la respuesta inmunitaria podría verse facilitada por determinadas herramientas de gestión, como los vallados o la alimentación suplementaria.

5. En el ciervo ibérico, la condición física, y la presencia de macroparásitos, (como garrapatas y *Elaphostrongylus cervi*) y microparásitos (como *Mycobacterium bovis*) están estadísticamente relacionados con determinados alelos del gen DRB3 del complejo mayor de histocompatibilidad.

6. Existe una expresión génica diferencial en linfonodos mesentéricos de ciervos infectados y no infectados con *Mycobacterium bovis*. Algunos de los genes sobreexpresados, como (CD74, gen asociado a la cadena no variable del MHC II), o inhibidos, como (IL2_R), sugieren que existen diversos mecanismos por los que esta bacteria sería capaz de modular y evitar la respuesta inmunitaria de su hospedador.

7. Finalmente, en esta tesis se comprueba que la compleja relación entre el ciervo y sus macro- y micro-parásitos, mediada por la respuesta inmunitaria viene determinada por complejos factores individuales, ambientales y genéticos.

## Agradecimientos

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